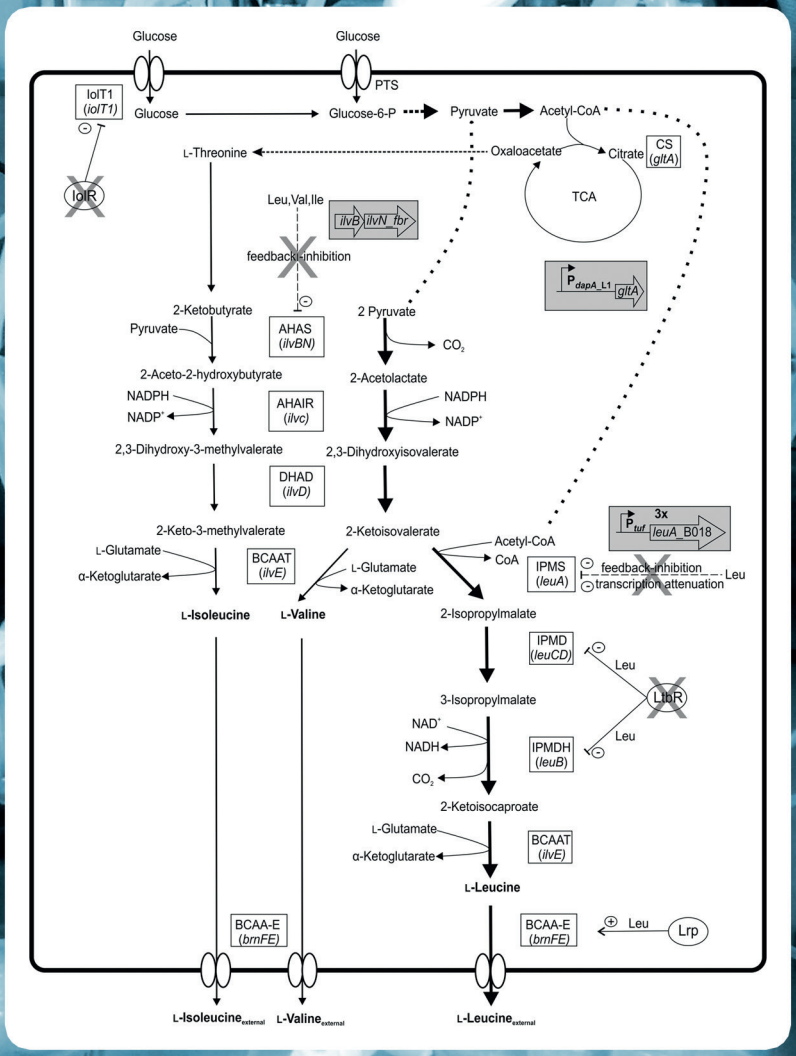


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Member of the Helmholtz Association

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# Content

## Abbreviations

| | |
|---|---|
| A | adenine or L-alanine |
| AHAIR | acetohydroxyacid isomeroreductase |
| AHAS | acetohydroxyacid synthase |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| BCAA(s) | branched-chain amino acid(s) |
| BHI(S) | brain heart infusion (sorbitol) |
| bidist. | bidistilled |
| *B. lactofermentum* | *Brevibacterium lactofermentum* |
| bp | base pair(s) |
| c | centi ($10^{-2}$) |
| C | cytosine |
| °C | degree Celsius |
| cDNA | complementary deoxyribonucleic acid |
| CDW | cell dry weight |
| *C. glutamicum* | *Corynebacterium glutamicum* |
| Δ | deletion (gene) |
| D | L-aspartic acid |
| dATP | deoxyadenosine triphosphate |
| DHAD | dihydroxyacid dehydratase |
| DNA | deoxyribonucleic acid |
| dCTP | deoxycytidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| dNTP | deoxynucleoside triphosphate |
| dTTP | deoxythymidine triphosphate |
| dUTP | deoxyuridine triphosphate |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| e.g. | *exempli gratia* (for example) |
| et al. | *et alii* (and others) |

| | |
|---|---|
| F | L-phenylalanine or farad |
| fbr | feedback-resistant |
| g | gram |
| *g* | standard gravity (9.81 m $s^{-1}$) |
| G | guanine or L-glycine |
| h | hour(s) |
| H | L-histidine |
| I | L-isoleucine |
| IPMS | 2-isopropylmalate synthase |
| IPMD | 3-isopropylmalate dehydratase |
| IPMDH | 3-isopropylmalate dehydrogenase |
| IPTG | isopropyl β-D-thiogalactoside |
| $Kan^r$ | kanamycin resistance |
| k | kilo ($10^3$) |
| kb | kilo base pairs |
| $K_i$ | affinity constant of inhibitor |
| $K_M$ | Michaelis constant |
| KIC | 2-ketoisocaproate |
| KIV | 2-ketoisovalerate |
| KMV | 2-keto-3-methylvalerate |
| l | liter |
| i.e. | *id est* (that is) |
| L | L-leucine |
| LB | Luria-Bertani |
| LtbR | leucine and tryptophane biosynthesis regulator |
| Lrp | leucine-responsive protein |
| m | milli ($10^{-3}$) or meter |
| M | molar (1 mole per litre) |
| min | minute(s) |
| mol | mole |
| MOPS | 3-morpholinopropanesulfonic acid |

| | |
|---|---|
| mRNA | messenger ribonucleic acid |
| *M. tuberculosis* | *Mycobacterium tuberculosis* |
| µ | micro ($10^{-6}$) |
| *µ* | growth rate |
| n | nano ($10^{-9}$) |
| N | L-asparagine |
| NAD(H) | nicotinamide adenine dinucleotide (reduced) |
| NADP(H) | nicotinamide adenine dinucleotide phosphate (reduced) |
| Ω | ohm |
| $OD_{600}$ | optical density at a wavelength of 600 nm |
| % | percent |
| P | L-proline |
| PCR | polymerase chain reaction |
| pH | negative decimal logarithmic value of hydrogen ion concentration |
| $pO_2$ | oxygen partial pressure |
| PTS | phosphotransferase system |
| R | L-arginine |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rRNA | ribosomal ribonucleic acid |
| RT | room temperature |
| sec | second(s) |
| T | thymine or L-threonine |
| Tris | tris(hydroxymethyl)aminomethane |
| tRNA | transfer ribonucleic acid |
| U | enzymatic activity (µmol per minute) |
| UV | ultraviolet |
| V | volt |
| v/v | volume per volume |
| wt | wild type |
| w/v | weight per volume |

## Summary

Due to the depletion of fossil energy sources, there is an increasing demand for alternative sustainable production processes utilizing renewable resources. Biotechnological approaches using microorganisms such as *Corynebacterium glutamicum* as biocatalysts play an important role for the production of beneficial substances. These include the three branched-chain amino acids L-valine, L-isoleucine, and L-leucine, as well as their respective keto acid precursors, which have diverse commercial applications in food, feed, and pharmaceutical industry. In this work, metabolic engineering of the *C. glutamicum* wild type was employed to develop efficient strains for the production of L-leucine and 2-ketoisocaproate.

The key-player enzyme in L-leucine biosynthesis is the *leuA*-encoded 2-isopropylmalate synthase which is feedback-inhibited by low L-leucine concentrations with a $K_i$ of 0.4 mM. A feedback-resistant variant of the 2-isopropylmalate synthase was identified and characterized biochemically in the available weak L-leucine producer B018, which had been obtained by random mutagenesis and screening. The respective gene *leuA*_B018, devoid of the attenuator region and under control of a strong promoter, was integrated in up to three copies into the genome of *C. glutamicum* wild type and combined with additional genomic modifications aimed at increasing L-leucine production. These modifications involved I) deletion of the gene *ltbR* encoding the repressor LtbR to increase expression of genes *leuBCD*, II) deletion of the gene *iolR* encoding the transcriptional regulator IolR to increase glucose uptake, III) reduction of citrate synthase activity to increase precursor supply, and IV) introduction of a modified *ilvN* gene encoding a feedback-resistant acetohydroxyacid synthase. The production performance of the resulting strains was characterized in shake flask and bioreactor cultivations. Under fed-batch conditions, the best producer strain accumulated L-leucine to levels exceeding the solubility limit of approximately 24 g $l^{-1}$. The maximal molar product yield and volumetric productivity were 0.30 mol per mol glucose and 4.3 mmol $l^{-1}$ $h^{-1}$, respectively. Moreover, the achieved values were obtained in a defined minimal medium with a prototrophic and plasmid-free strain, making this process highly interesting for industrial application.

Based on the described L-leucine producers, strains for the production of the keto acid 2-ketoisocaproate, the direct precursor of L-leucine, were constructed and characterized as well. This was achieved by deletion of the gene encoding the transaminase IlvE or replacement of the ATG start codon of *ilvE* by GTG start codon. The resulting strains accumulated up to 6.1 g $l^{-1}$ 2-ketoisocaproate in shake flask cultivations, with a molar product yield of 0.20 mol per mol glucose and a volumetric productivity of 1.4 mmol $l^{-1}$ $h^{-1}$.

## Zusammenfassung

Angesichts knapper werdender fossiler Rohstoffe erlangen alternative nachhaltige Produktionsprozesse unter Verwendung erneuerbarer Ressourcen zunehmend an Bedeutung. Biotechnologische Ansätze, die Mikroorganismen wie *Corynebacterium glutamicum* als Biokatalysatoren verwenden, spielen dabei eine wichtige Rolle zur Produktion nützlicher Substanzen. Hierzu gehören auch die drei verzweigtkettigen Aminosäuren L-Valin, L-Isoleucin und L-Leucin und ihre Ketosäure-Vorstufen. Diese Substanzen haben verschiedenste Anwendungen in der Nahrungsmittel-, Futtermittel- und Pharmaindustrie. Im Rahmen dieser Arbeit wurden, ausgehend vom *C. glutamicum*-Wildtyp, mittels *metabolic engineering* effiziente Produktionsstämme für L-Leucin und 2-Ketoisocaproat entwickelt.

Das Schlüsselenzym der L-Leucin-Biosynthese ist die 2-Isopropylmalatsynthase, kodiert durch das *leuA*-Gen, die in *C. glutamicu*m durch geringe L-Leucin-Konzentrationen *feedback*-inhibiert wird ($K_i$ = 0,4 mM). In einem zur Verfügung stehenden schwachen L-Leucin-Produzenten B018, der durch Zufallsmutagenese und *Screening* erhalten worden war, konnte eine *feedback*-resistente Variante der 2-Isopropylmalatsynthase identifiziert und biochemisch charakterisiert werden. Das entsprechende Gen *leuA*_B018 wurde ohne Attenuatorregion und unter Kontrolle eines starken Promoters in bis zu drei Kopien in das Genom von *C. glutamicum* eingebracht. Daneben wurden weitere genomische Modifikationen durchgeführt, um die L-Leucin-Produktion zu erhöhen: I) Deletion des *ltbR*-Gens für den Repressor LtbR zur Erhöhung der Expression der Gene *leuBCD*, II) Deletion des *iolR*-Gens für den Transkriptionsregulator IolR zur Erhöhung der Glucose-Aufnahmerate, III) Reduktion der Citratsynthase-Aktivität zur Erhöhung der Vorstufenbereitstellung, sowie IV) Einführung eines modifizierten *ilvN*-Gens für eine *feedback*-resistente Acetohydroxysäuresynthase. Die Produktionsleistung der konstruierten Stämme wurde in Schüttelkolben und Bioreaktoren charakterisiert. In *fed-batch*-Fermentationen akkumulierte der beste Stamm L-Leucin-Konzentrationen über die Löslichkeitsgrenze von 24 g $l^{-1}$ hinaus. Die maximalen Werte für molare Produkt-Ausbeute und volumetrische Produktivität betrugen 0,30 mol pro mol Glukose beziehungsweise 4,3 mmol $l^{-1}$ $h^{-1}$. Da diese Werte in definiertem Minimalmedium unter Verwendung eines prototrophen und Plasmid-freien Stamms erreicht wurden, ist der beschriebene Prozess sehr interessant für die industrielle Anwendung.

Basierend auf den beschriebenen L-Leucin-Produzenten wurden auch Stämme für die Produktion von 2-Ketoisocaproat, der direkten Ketosäure-Vorstufe von L-Leucin, konstruiert und charakterisiert. Dazu wurde entweder das Gen für die Transaminase IlvE deletiert oder das ATG-Startcodon von *ilvE* gegen ein GTG-Startcodon ausgetauscht. Mit diesen Stämmen konnten bis zu 6,1 g $l^{-1}$ 2-Ketoisocaproat in Schüttelkolben-Kultivierungen produziert werden, mit einer molaren Produkt-Ausbeute von 0,20 mol pro mol Glukose und einer volumetrischen Produktivität von 1,4 mmol $l^{-1}$ $h^{-1}$.

# 1 Introduction

## 1.1 Bioeconomy: Sustainable production processes using renewable resources

In times of the depletion of fossil energy sources, society is facing steadily increasing oil and gas prices, proceeding environmental pollution, and climate change. In addition, the rapid growth of the world population increases the demand for energy, which will further exacerbate the economic and social challenges in the near future. Therefore, the focus of bulk and fine chemical production is shifting more and more from products based on fossil fuels to production processes using renewable resources. Bioeconomy strategies have been elaborated in different nations and regions, i.e. the USA and European Union (Philp et al., 2013). The term “bioeconomy” comprises the efforts to create sustainable economic growth based on renewable and environmentally friendly products. Biotechnology, especially white biotechnology, is expected to take a major part in this development with biotransformations and fermentations (Octave and Thomas, 2009).

As an alternative to classical chemical synthesis processes, biotechnology offers applications for the ecological and economic valuable production of bulk chemicals, i.e. fuels, solvents, plastics, etc., from renewable plant biomass. Microorganisms are employed as biocatalysts in fermentation processes to produce beneficial substances such as amino acids, organic acids, alcohols, vitamins, or antibiotics in an efficient and cost-effective manner (Wendisch et al., 2006a). The market share of biotechnological products concerning bio-based chemicals is expected to grow worldwide from currently 9-13% to 22-28% in 2025 (Kircher, 2012).

Due to its economic and ecological advantages in comparison to chemical methods such as synthesis and extraction, the biotechnological production of amino acids has shown a rapid development and represents one of the most important categories of fermentation products (Leuchtenberger et al., 2005). To establish optimized, economic, and competitive biotechnological processes, factors like bioreactor design, optimization of yields, reduction of process times, and downstream processing have to be addressed. Especially the latter point dealing with extraction and purification of the desired products is a very important factor (Schügerl and Hubbuch, 2005). Nevertheless, suitable production strains with desired properties for the respective substances to be produced are absolutely crucial and represent the core of fermentation processes (Hermann, 2003). Therefore, strain development for biotechnological purposes has a long tradition. Besides *Escherichia coli*, *Corynebacterium glutamicum* is one of the most important bacteria used in industrial biotechnology.

## 1.2 Biotechnological relevance of *C. glutamicum* and applications of branched-chain amino acids

*C. glutamicum*, a gram-positive soil bacterium, has become a platform organism in industrial biotechnology, especially for the production of amino acids. Its biosynthesis capacities and its GRAS ("generally recognized as safe") status make it an ideal host for the effective and safe production of a wide variety of compounds. *C. glutamicum* is able to grow on diverse renewable carbon sources as feedstock. In particular, sugars from agricultural crops such as cane molasses, beet molasses, as well as starch molasses from corn and cassava are used in industrial fermentations (Ikeda, 2012). Feedstock variability is an important economic factor since market prices of carbon sources are highly volatile (Kircher, 2012). To broaden the substrate spectrum, efforts are being made to engineer *C. glutamicum* for the utilization of alternative feedstock, i.e. starch, hemicellulose, and glycerol (Becker and Wittmann, 2012a; Wendisch et al., 2006a). Since the genome of *C. glutamicum* is known (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) and efficient techniques for its genetic engineering exist (Binder et al., 2013; Kirchner and Tauch, 2003), the creation of tailor-made strains with desired properties is feasible. Moreover, this microorganism has additional advantages in comparison to other hosts: For example, the reduced abundance of isoenzymes enables efficient metabolic flux control. This and the increased phage-resistance as compared to *E.coli* make *C. glutamicum* an ideal candidate for engineering and production. Moreover, no deleterious accumulation of acetic acid occurs and the reduced presence of degradative enzymes for amino acids facilitates efficient production of these substances (Eggeling et al., 1997).

*C. glutamicum* is mainly used to produce several million tons of amino acids annually, with the flavor enhancer L-glutamate (2,400,000 tons/year) and the feed additive L-lysine (1,500,000 tons/year) as major products (Becker and Wittmann, 2012b). Three comprehensive monographs (Burkovski, 2008; Eggeling and Bott, 2005; Yukawa and Inui, 2013) depict the increasing knowledge obtained about this bacterium. Apart from amino acids, strains have also been developed for the production of other commercially interesting compounds (Becker and Wittmann, 2012a), such as organic acids (Litsanov et al., 2012a, 2012b; Okino et al., 2008; Wieschalka et al., 2013), diamines (Kind and Wittmann, 2011; Mimitsuka et al., 2007; Schneider and Wendisch, 2011), or alcohols (Blombach et al., 2011; Inui et al., 2004; Smith et al., 2010;). Moreover, also the production of heterologous proteins is possible using this host (Scheele et al., 2013, and references therein).

Besides the major products L-glutamate and L-lysine, *C. glutamicum* can also be used for the production of many other amino acids. These include also the group of branched-chain amino acids (BCAAs), i.e. L-valine, L-isoleucine, and L-leucine. These amino acids have a small but substantially growing market of up to 5,000 tons per year (Becker and Wittmann,

2012b). BCAAs are the most abundant of the essential amino acids, are hydrophobic due to their unsubstituted aliphatic chains with branched alkyl groups (Figure 1), and have diverse applications (Pátek, 2007, Yoshizawa, 2012). They are used as components of pharmaceuticals and cosmetics, in the animal feed industry, as dietary products, and as precursors in the chemical synthesis of herbicides (Park and Lee, 2010a). BCAAs are used, for example, in the medical treatment of patients with hepatic diseases to improve the nutritional status (Freund et al., 1982). They are absolutely essential for immune cell functions such as lymphocyte responsiveness (Calder, 2006). During endurance sports events, these amino acids, particularly L-leucine, attenuate the breakdown of muscle proteins, promote protein synthesis in skeletal muscle, and prevent premature muscle fatigue (Rennie et al., 2006; Shimomura et al, 2006). Therefore, BCAAs are used as special dietary supplements in sports nutrition because they are expected to promote maintaining and regeneration of the skeletal muscle (Pátek, 2007). L-isoleucine is particularly used in the food and feed industry (Morbach et al., 2000). Studies showed that L-isoleucine stimulates glucose uptake in the muscle, promotes whole body oxidation, and derepresses gluconeogenesis (Yoshizawa, 2012). L-Valine has applications also as feed additive and precursor for the chemical synthesis of herbicides (Eggeling, 1996; Leuchtenberger, 1996).

The BCAA L-leucine is the focus of this work. Together with L-valine and L-isoleucine, it is used as a component of infusion solutions and special dietary products (Eggeling et al., 1997). L-Leucine plays a potent role in the stimulation of muscle protein synthesis, while the other BCAAs are less effective, and mechanisms for its signaling action are discussed in the literature (Garlick, 2005; Yoshizawa, 2012). Also the effect of this amino acid on glucose homeostasis has been investigated (Layman, 2003). L-Leucine is also applied as a flavoring substance and as a lubricant for tablet production (Leuchtenberger, 1996; Rotthäuser et al., 1998).

The direct keto acid precursors of L-valine, L-isoleucine, and L-leucine are 2-ketoisovalerate (KIV), 2-keto-3-methylvalerate (KMV), and 2-ketoisocaproate (KIC), respectively (Figure 1). These keto acids also have a variety of applications similar to their corresponding BCAAs in the medical, biological, and food area since they play an important role in living organisms as regulation factors in metabolism and key intermediates of biosynthesis (Bückle-Vallant et al., 2014; Krause et al., 2010; Zhu et al., 2011). They are, for example, used in the therapy of chronic kidney disease patients (Aparicio et al., 2012). KIC has similar effects as its corresponding amino acid L-leucine because it has anti-catabolic properties through inhibition of muscle proteolysis and provokes enhancement of protein synthesis, especially in the skeletal muscle (Escobar et al., 2010; Zanchi et al., 2011). Also, an insulin-releasing action of KIC (Heissig et al., 2005) and its inhibitory effect on glucagon release (Leclercqmeyer et al., 1979) have been discussed. Furthermore, the use of KIC and

the other keto acids as precursors for other interesting products is feasible. For example, it was shown that KIC can serve as a basis for the production of the biofuel isopentanol (3-methyl-1-butanol) (Cann and Liao, 2010). Until now, KIV, KMV, and KIC are mainly produced by chemical synthesis, using harsh reaction conditions and multiple purification steps (Cooper et al., 1983), but first microbial producers for KIV and KIC have been reported recently (Bückle-Vallant et al., 2014; Krause et al., 2010).

All these mentioned aspects provide promising applications. Therefore, BCAAs and their keto acid precursors are interesting products of industrial fermentations. In order to understand the processes that lead to the accumulation of these compounds in production strains, detailed knowledge of their biosynthesis pathways is necessary.

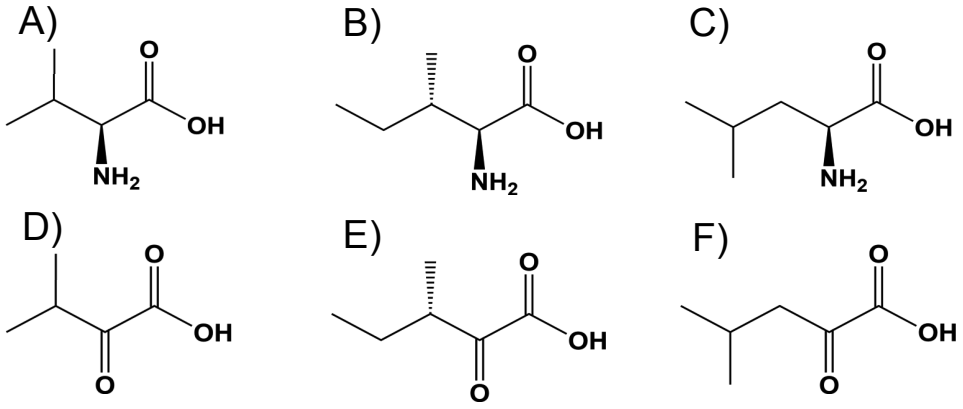



**Figure 1. The three proteinogenic branched-chain amino acids L-valine (A), L-isoleucine (B), and L-leucine (C), as well as their respective keto acid precursors 2-ketoisovalerate (D), 2-keto-3-methylvalerate (E), and 2-ketoisocaproate (F).**

## 1.3 Biosynthesis of BCAAs and its regulatory mechanisms in *C. glutamicum*

The biosynthesis pathways of the BCAAs (Figure 2) are overlapping and interrelated since they are characterized by homologous reactions catalyzed by single enzymes, branch points, as well as multivalent regulation of both gene expression and enzyme activity (Pátek, 2007).

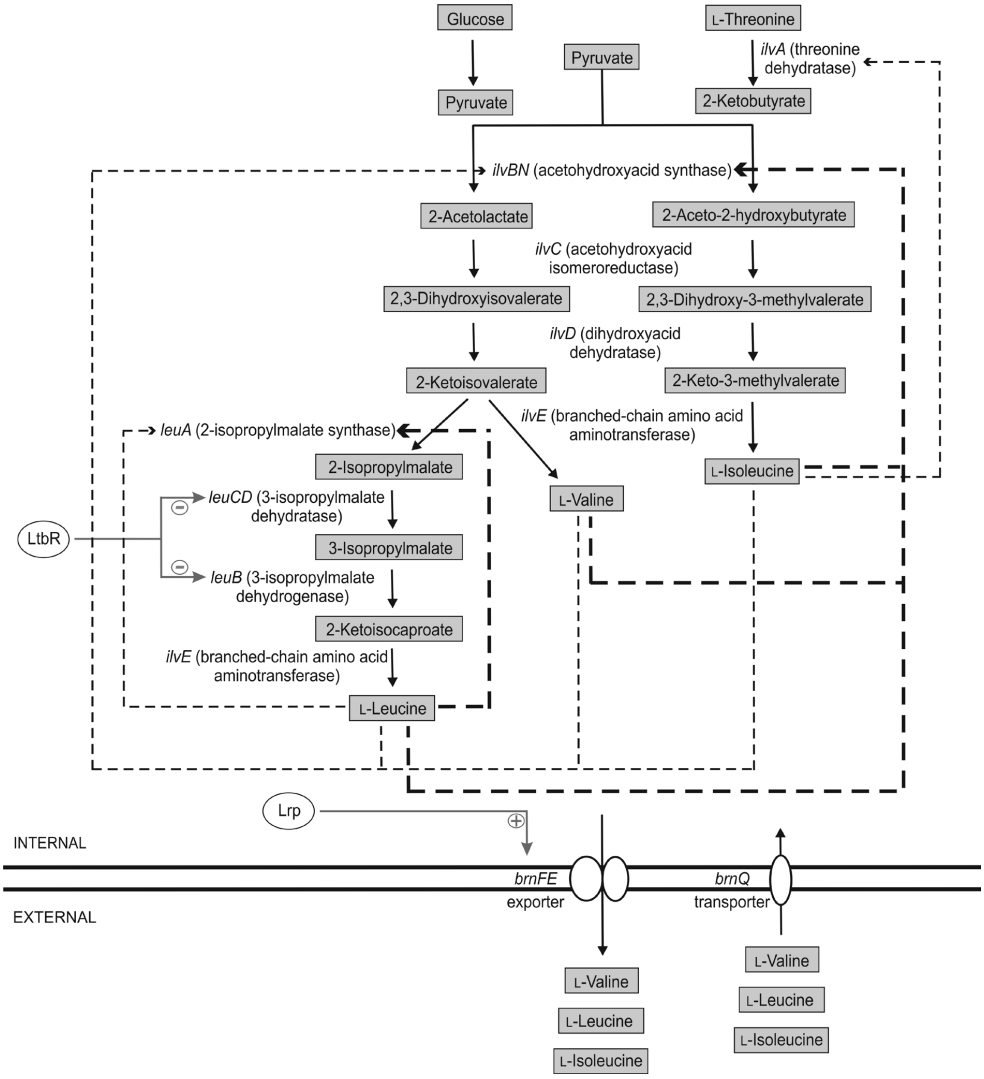



**Figure 2. The biosynthetic pathways of BCAAs, as well as corresponding regulation mechanisms and transport systems involved in *C. glutamicum* (taken and modified from Park and Lee, 2010a).**
Dashed lines indicate feedback-inhibition. The thick dashed lines indicate transcriptional attenuation regulation. Gray lines indicate regulation of gene expression by Lrp and LtbR; "+" and "-" indicate activation and repression of gene expression, respectively. The *avtA* gene encoding the transaminase AvtA (Marienhagen et al., 2005) is not depicted.

As the first step in the biosynthesis of L-valine and L-leucine in *C. glutamicum*, two molecules of pyruvate are converted to 2-acetolactate by the enzyme acetohydroxyacid synthase (AHAS), encoded by the genes *ilvB* (coding for the catalytic subunit) and *ilvN* (coding for the regulatory subunit). 2-Acetolactate is further metabolized to

2,3-dihydroxyisovalerate and 2-ketoisovalerate (KIV) by the enzymatic activities of *ilvC*-encoded acetohydroxyacid isomeroreductase (AHAIR) and *ilvD*-encoded dihydroxyacid dehydratase (DHAD), respectively (Cordes et al., 1992; Keilhauer et al., 1993; Radmacher et al., 2002). The genes *ilvB*, *ilvN* and *ilvC* are organized as an operon and their expression is controlled by a transcriptional attenuation mechanism mediated by all three BCAAs (Morbach et al., 2000). In parallel reactions, AHAS, AHAIR, and DHAD also catalyze the subsequent formation of 2-aceto-2-hydroxybutyrate, 2,3-dihydroxy-3-methylvalerate, and finally 2-keto-3-methylvalerate (KMV) from pyruvate and 2-ketobutyrate. The latter substrate is formed by action of *ilvA*-encoded threonine dehydratase from L-threonine. This is the only enzyme specific for L-isoleucine biosynthesis and exhibits allosteric feedback-inhibition by L-isoleucine (Cordes et al., 1992, Möckel et al., 1992). It was shown that the enzymatic activity of AHAS is inhibited by any of the three branched-chain amino acids (Eggeling et al., 1987), but inhibition of AHAS does not exceed 57%, even in the presence of all three BCAAs (Elišáková et al., 2005). The half maximal inhibitory concentration ($IC_{50}$) values of AHAS for L-valine, L-isoleucine, and L-leucine are 0.9 mM, 3.1 mM, and 6.0 mM, respectively (Leyval et al., 2003). KMV is transaminated to L-isoleucine by transaminase IlvE. KIV is transaminated to L-valine by IlvE, but also by the transaminase AvtA (Marienhagen et al., 2005; Marienhagen and Eggeling, 2008).

Furthermore, KIV is a precursor for D-pantothenate biosynthesis and a substrate of the 2-isopropylmalate synthase (IPMS). This enzyme encoded by *leuA* represents the first step in the specific L-leucine-biosynthesis pathway (Pátek et al., 1994). Gene *leuA* is controlled by an attenuation mechanism which reduces expression to approximately 50% in the presence of L-leucine. In addition, IPMS underlies the control of a strong feedback-inhibitory mechanism by L-leucine ($K_i$ = 0.4 mM) (Mentz et al., 2013; Pátek et al., 1994). This enzyme synthesizes 2-isopropylmalate from one molecule of KIV and one molecule of acetyl-CoA. Subsequently, 2-isopropylmalate is converted to 3-isopropylmalate and finally to the direct L-leucine precursor KIC. This is performed by the enzymatic activities of 3-isopropylmalate dehydratase (IPMD) and 3-isopropylmalate dehydrogenase (IPMDH), which are encoded by *leuCD* and *leuB*, respectively (Pátek et al., 1994). Both *leuCD* and *leuB* are repressed by the IclR-type transcriptional regulator LtbR (leucine and tryptophane biosynthesis regulator) in response to L-leucine (Brune et al., 2007). Eventually, KIC is transaminated to L-leucine by the transaminase IlvE. Besides that, other transaminases such as the *aroT*-encoded aromatic amino acid transaminase exhibit weak side activities for the transamination of KIC to L-leucine (Marienhagen et al., 2005).

All three BCAAs as well as L-methionine are excreted by a two component permease of the LIV-E family, encoded by *brnFE* (Kennerknecht et al., 2002). Expression of *brnFE* is thereby activated via the transcriptional regulator Lrp (leucine-responsive protein) by the

BCAAs and L-methionine, but mainly in the presence of elevated intracellular L-leucine concentrations (Lange et al., 2012). The transporter encoded by *brnQ* imports all three BCAAs (Tauch et al., 1998).

## 1.4 *C. glutamicum* strains for the production of BCAAs and their keto acid precursors: Metabolic engineering as an alternative to random mutagenesis

Production strains of *C. glutamicum* and *E. coli* for BCAAs have been developed based on random mutagenesis, but also strains exist that have been constructed by targeted metabolic engineering (Park and Lee, 2010a). Effective strains have already been created from the *C. glutamicum* wild type for production of BCAAs, which is less the case for *E. coli*, and this work will also focus on *C. glutamicum*. The classical approach to generate microbial producers comprises random mutagenesis and selection procedures until strains are obtained exhibiting desired yield and productivity. This strategy created potent production strains for all three BCAAs in the past (Park and Lee, 2010a). Nevertheless, random mutagenesis has serious drawbacks: Random distributions of mutations not related to the targeted pathway can cause undesired changes in cell physiology, e.g. high by-product accumulation and growth retardation. Moreover, “dead-end” mutants are often produced which cannot be improved further (Park and Lee, 2010b). In contrast to this, rational metabolic engineering offers the possibility to create genetically defined strains. Disadvantages of the classical approaches can be prevented and desired properties, e.g. prevention of auxotrophies, can be directly addressed. Nevertheless, strains obtained by undirected mutagenesis offer a rich source of mutations which increase product formation and therefore are used in metabolic engineering (Ikeda et al., 2006) (Figure 3).

Using recombinant DNA technology, metabolic engineering is defined as the direct improvement of cellular properties through modification of specific biochemical reactions (Stephanopoulos, 1999). This includes the targeted amplification or deletion of DNA sequences as well as the modulation and deregulation of gene expression or enzyme activity. In this manner, carbon flux is optimally directed to the formation of the target metabolite in order to achieve yields near the theoretical maximum values (Park and Lee, 2010b). In the case of the production of BCAAs, their interrelated biosynthesis (section 1.3) makes metabolic engineering particularly challenging. Modifications in one BCAA pathway can easily lead to detrimental effects in the other pathways or to large accumulation of by-products, which would negatively affect yield and downstream processing. Selected existing

strains for the production of the three BCAAs and their keto acid precursors are described in this section and comprise different metabolic engineering strategies, such as amplification or deletion of target genes, modulation of gene expression via promoter exchange, and elimination of regulatory mechanisms.

For L-valine production, suitable strains of *Brevibacterium lactofermentum*, which was later renamed into *C. glutamicum* (Liebl et al., 1991), were achieved by random mutagenesis (Tsuchida et al., 1975). There are also several *C. glutamicum* strains that have been constructed rationally. The first rationally designed strain was reported by Radmacher et al. (2002). In this strain, deletion of the genes *panBC*, involved in the formation of D-pantothenate from KIV, and deletion of *ilvA*, together with plasmid-borne overexpression of *ilvBNCD,* led to L-valine accumulation. Blombach et al. (2007) achieved L-valine production by deleting the *aceE* gene coding for E1p enzyme of the pyruvate dehydrogenase complex (PDHC) and plasmid-based overexpression of *ilvBNCE*. This strain was improved by additional deletion of *pqo,* encoding pyruvate:quinone oxidoreductase, and *pgi*, encoding phosphoglucose isomerase (Blombach et al., 2008). A similar improved strain was obtained by downregulation of *aceE* expression via promoter exchange in a Δ*pqo Δppc* (the latter encoding phosphoenolpyruvate carboxylase) background and plasmid-based overexpression of *ilvBNCE* (Buchholz et al., 2013). Besides overexpressing *ilvBNC* on a plasmid in a Δ*ilvA* Δ*panB* strain, Elišáková et al. (2005) made use of an *ilvN* allele (*ilvNM13*) resulting in an AHAS variant that is feedback-resistant for all three BCAAs.

For L-isoleucine production, Kase and Nakayama (1977) reported a *C. glutamicum* strain that was obtained by a classical random mutagenesis approach based on an L-threonine-producer, but also rationally designed strains exist. Using a *C. glutamicum* L-lysine producer with feedback-resistant aspartate kinase, encoded by *lysC*(fbr), L-isoleucine accumulation was achieved by three *hom*(fbr) copies, encoding feedback-resistant homoserine dehydrogenase, and *ilvA*(fbr), encoding feedback-resistant threonine dehydratase, on a multicopy plasmid (Morbach et al., 1995). In a subsequent study, transport of L-isoleucine was identified as a major limiting step for the production of this amino acid (Morbach et al. 1996). Indeed, Xie et al. (2012) showed that deletion of the *brnQ*-encoded BCAA importer and, more importantly, overexpression of the *brnFE*-encoded BCAA exporter increased L-isoleucine production in the *C. glutamicum* strain YILW. Plasmid-based co-expression of a feedback-resistant threonine dehydratase and acetohydroxyacid synthase in *C. glutamicum* JHI3-156 led to an increase of L-isoleucine production (Yin et al., 2012).

Only a few studies deal with the microbial production of L-leucine using either *B. lactofermentum* (respectively *C. glutamicum*) or *E. coli* as hosts (Park and Lee, 2010a). All *C. glutamicum*-based production strains were created by random mutagenesis: One of the first L-leucine producers was a 2-thiazolealanine-resistant, methionine-isoleucine-auxotrophic

mutant derived from the L-glutamic acid producing *B. lactofermentum* 2256 by nitrosoguanidine treatment (Tsuchida et al., 1974). The productivity of this strain was further optimized by additional mutagenesis steps (Ambe-Ono et al., 1996; Tsuchida and Momose, 1986). Another *S*-(2-aminoethyl)-L-cysteine-resistant mutant was derived from *C. glutamicum* ATCC 13032 (Azuma et al., 1987).

Besides a described biotransformation with *Rhodococcus opacus* using L-leucine as substrate for the production of KIC (Zhu et al., 2011), first *C. glutamicum* strains for the production of KIV and KIC have been described recently in the literature. A first KIV producing *C. glutamicum* strain was achieved by deleting the genes *aceE*, *ilvE*, and *pqo* and plasmid-based overexpression of *ilvBNCD* (Krause et al., 2010). Based on this work, an improved producer of KIV was constructed by downregulating the activity of the *aceE* promoter in a Δ*ilvE* Δ*pqo* Δ*ppc* strain background and additional plasmid-based overexpression of *ilvBNCD* (Buchholz et al., 2013). Very recently, Bückle-Vallant and colleagues (2014) designed a *C. glutamicum* strain for the production of KIC. They downregulated the activity of citrate synthase via a promotor exchange based on a promoter originally used to improve L-lysine synthesis (van Ooyen et al., 2012). Furthermore, this strain is characterized by deletions of the genes *ilvE*, *ltbR*, *prpC1*, and *prpC2* (the latter two encoding methylcitrate synthases) as well as plasmid-based overexpression of *ilvBNCD* and a *leuA* allele from *E. coli* encoding a feedback-resistant 2-isopropylmalate synthase.

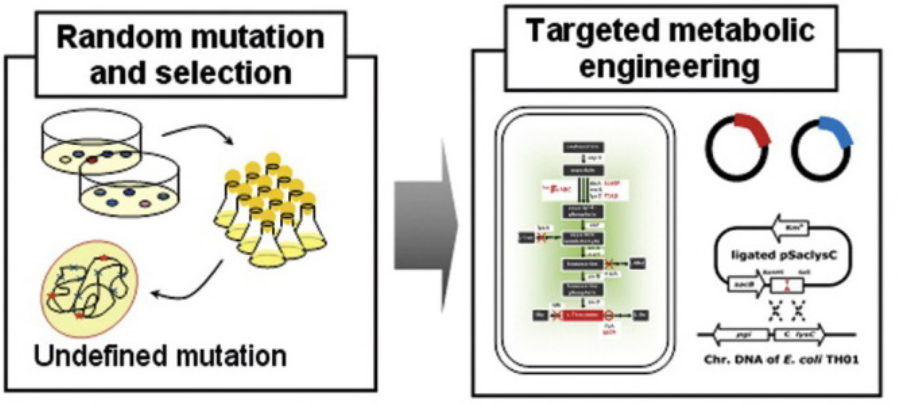



**Figure 3. Strain development by random mutagenesis/selection and targeted metabolic engineering (from Park and Lee, 2008, modified).**
The classical method of strain development comprises random mutagenesis with subsequent selection procedures. This strategy is repeated until the desired properties, such as high product titers, are achieved. Alternatively, targeted metabolic engineering of genes and biosynthesis pathways has become a standard strategy for strain development. The combined use of random and directed approaches is recognized as an efficient method for strain construction (Klein-Marcuschamer and Stephanopoulos, 2010).

## 1.5 Aims of this work

This work describes the rational design and construction of *C. glutamicum* L-leucine production strains by targeted metabolic engineering starting from the wild-type strain. The aim was to design genetically defined producers that reach high values in terms of product titer, yield, and productivity. Moreover, the created strains should be devoid of auxotrophies, plasmids and heterologous genes. Auxotrophies have the disadvantage that supplements have to be added to the fermentation medium in order to allow microbial growth, which increases costs of the fermentation process. Due to their antibiotic resistance markers, the industrial application of plasmids in genetically engineered production strains in food and feed industry can be resisted by regulatory agencies (Tauch et al., 2002). This is also true for the use of heterologous genes, which are not accepted by the society in large-scale fermentations. Besides that, the absence of plasmids, markers, and heterologous genes often provides more stable producer strains (Pátek, 2007). Addressing all these aspects was supposed to facilitate a direct use of the constructed strains for industrial fermentation processes. The single metabolic steps should be analyzed and discussed concerning their effects on L-leucine production and strategies should be developed for further strain improvement. Moreover, possibilities of turning existing L-leucine producers into producers for the precursor 2-ketoisocaproate, which also has interesting commercial applications, were addressed.

# 2 Material and Methods

## 2.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this work as well as their relevant characteristics are listed in Table 1 and Table 2, respectively.

Plasmid-based gene expressions were achieved using vector pAN6 carrying an IPTG-inducible *tac* promoter (Frunzke et al., 2008) or derivatives of pJC1 (Cremer et al., 1990). All marker-free in-frame deletions and integrations of DNA sequences in *C. glutamicum* strains were done via two-step homologous recombination using vector pK19*mobsacB* (Schäfer et al., 1994). Plasmid constructions are described in section 2.5.14.

**Table 1.** Bacterial strains used in this work.

| Strain | Relevant characteristics | Source or reference |
|---|---|---|
| ***C. glutamicum* strains** | | |
| Wild type | wild type ATCC 13032 (biotin auxotrophic) | Abe et al. (1967) |
| B018 | L-leucine producing *C. glutamicum* strain created by random mutagenesis | Amino GmbH, Frellstedt, Germany |
| Δ*ltbR* | ATCC 13032 derivative with in-frame deletion of *ltbR* | This work |
| Δ*ltbR* Δ*leuA* | *C. glutamicum* Δ*ltbR* derivative with in-frame deletion of *leuA* along with its 180 bp upstream region | This work |
| Δ*ltbR ilvN*_fbr | *C. glutamicum* Δ*ltbR* derivative with chromosomally integrated mutations into *ilvN* coding for amino acid exchanges G20D, I21D, and I22F | This work |
| Δ*ilvE* | ATCC 13032 derivative with in-frame deletion of *ilvE* | Marienhagen et al. (2005) |

| Strain | Relevant characteristics | Source or reference |
|---|---|---|
| MV-Leu7 | *C. glutamicum* Δ*ltbR* derivative with a mutated *leuA* gene encoding the amino acid exchanges R529H and G532D (*leuA*_B018) in comparison to the wild-type *leuA* | This work |
| MV-Leu20 | *C. glutamicum* Δ*ltbR* Δ*leuA* derivative with chromosomal integration of *leuA* from B018 under control of the *tuf* promoter into the Δ*leuA* locus | This work |
| MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 | MV-Leu20 derivative with chromosomal integration of a second copy of *leuA* from B018 under control of the *tuf* promoter into the Δ*ltbR* locus | This work |
| MV-ValLeu33 | MV-Leu20 derivative with chromosomally integrated mutations into *ilvN* coding for amino acid exchanges G20D, I21D, and I22F | This work |
| MV-Leu55 | MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 derivative with chromosomally integrated mutations into *ilvN* coding for amino acid exchanges G20D, I21D, and I22F | This work |
| MV-Leu55 Δ*brnQ* | MV-Leu55 derivative with in-frame deletion of *brnQ* | This work |
| MV-Leu55 Δ*avtA* | MV-Leu55 derivative with in-frame deletion of *avtA* | This work |
| MV-Leu55 Δ*iolR* | MV-Leu55 derivative with in-frame deletion of *iolR* | This work |
| MV-Leu55 Δcg3022 | MV-Leu55 derivative with in-frame deletion of cg3022 | This work |

| Strain | Relevant characteristics | Source or reference |
|---|---|---|
| MV-LeuF1 | MV-Leu55 Δ*iolR* derivative with chromosomal integration of a third copy of *leuA* from B018 under control of the *tuf* promoter integrated into the intergenic region between cg1121 and cg1122 | This work |
| MV-LeuF2 | MV-LeuF1 derivative with chromosomal replacement of the *gltA* promoter by *dapA*-L1 promoter | This work |
| MV-KICF1 | MV-LeuF1 derivative with chromosomal replacement of the ATG start codon of *ilvE* by GTG start codon | This work |
| SH-KIC20 | MV-Leu20 derivative with chromosomal replacement of the ATG start codon of *ilvE* by GTG start codon | Sabine Haas (FZ Jülich, Germany) |
| ***E. coli* strains** | | |
| DH5α | $F^-$ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 ($r_K^-$, $m_K^+$) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1 | Invitrogen (Karlsruhe, Germany) |

**Table 2.** Plasmids used in this work.

| Plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| pAN6 | Kan$^r$; *E. coli*/*C. glutamicum* shuttle vector for inducible gene expression ($P_{tac}$, *lacI*$^q$, pUC18 *ori*$V_{E.coli}$, pBL1 *ori*$V_{C.glutamicum}$) | Frunzke et al. (2008) |
| pAN6-*leuA*_wt | Kan$^r$; pAN6 derivative containing the *leuA* gene from *C. glutamicum* wild type under control of the *tac* promoter | This work |

| Plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| pAN6-*leuA*_B018 | Kan$^r$; pAN6 derivative containing the *leuA* gene from B018 under control of the *tac* promoter | This work |
| pAN6-*leuA*_mod | Kan$^r$; pAN6 derivative containing the *leuA*_mod gene under control of the *tac* promoter | This work |
| pK19*mobsacB* | Kan$^r$; vector for allelic exchange in *C. glutamicum* (pK18 *oriV*$_{E.coli}$, *sacB*, *lacZ*α) | Schäfer et al. (1994) |
| pK19*mobsacB*-*leuA*_B018_BS | Kan$^r$, pK19*mobsacB* derivative containing a 1378 bp overlap-extension PCR product (SbfI/XbaI) which covers the partial sequence of *leuA* from B018 coding for the amino acid exchanges R529H and G532D flanked by its upstream and downstream regions (approximately 560 bp and 810 bp, respectively) | This work |
| pK19*mobsacB*-*ΔleuA* | Kan$^r$, pK19*mobsacB* derivative containing a 1060 bp overlap-extension PCR product (flanking SbfI/XbaI and intrinsic NdeI/MluI/SpeI restriction sites) which covers the flanking upstream and downstream regions (approximately 490 bp and 550 bp, respectively) of the *leuA* gene along with its 180 bp upstream region | This work |
| pK19*mobsacB*-P$_{tuf}$-*leuA*_B018 | Kan$^r$, pK19*mobsacB*-*ΔleuA* derivative containing the *leuA* gene from B018 (MluI/SpeI) under control of the *tuf* promoter (NdeI/MluI) flanked by upstream and downstream regions of *leuA* | This work |
| pK19*mobsacB*-Δ*ltbR* | Kan$^r$, pK19*mobsacB* derivative for in-frame deletion of gene *ltbR* | Jan van Ooyen (FZ Jülich, Germany) |
| pK19*mobsac*B-Δ*ilvE* | Kan$^r$, pK19*mobsacB* derivative for in-frame deletion of gene *ilvE* | Marienhagen et al. (2005) |

| Plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| pK19*mobsacB-ΔltbR*::$P_{tuf}$-*leuA*_B018 | Kan$^r$, pK19*mobsacB-ΔltbR* derivative containing the *leuA* gene from B018 under control of the *tuf* promoter along with the 70 bp upstream region of *leuA* (AvrII) covered by upstream and downstream regions of *ltbR* | This work |
| pK19*mobsacB-ilvN*_fbr | Kan$^r$, pK19*mobsacB* derivative containing a 1279 bp overlap-extension PCR product (SbfI/XbaI) which covers the partial sequence of *ilvN* coding for the amino acid exchanges G20D, I21D, and I22F flanked by its upstream and downstream regions (approximately 620 bp and 660 bp, respectively) | This work |
| pK19*mobsacB-ΔbrnQ* | Kan$^r$, pK19*mobsacB* derivative for in-frame deletion of gene *brnQ* | Kennerknecht (2003) |
| pK19*mobsacB-ΔavtA* | Kan$^r$, pK19*mobsacB* derivative for in-frame deletion of gene *avtA* | Marienhagen et al. (2005) |
| pK19*mobsac-ΔiolR2* | Kan$^r$, pK19*mobsacB* derivative for in-frame deletion of gene *iolR* | Klaffl et al. (2013) |
| pK19*mobsac*-Δ$P_{gltA}$::$P_{dapA\text{-}L1}$ | Kan$^r$, pK19*mobsacB* derivative for chromosomal replacement of the *gltA* promoter by *dapA*-L1 promoter | van Ooyen et al. (2012) |
| pK19*mobsac*-Δcg3022 | Kan$^r$, pK19*mobsacB* derivative containing a 1171 bp overlap-extension PCR product (SbfI/EcoRI) which covers the flanking upstream and downstream regions (approximately 570 bp and 510 bp, respectively) of gene cg3022 | This work |

| Plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| pK18*mobsac*-lysOP7 | Kan$^r$, contains artificial operon integrated into the intergenic region between cg1121 and cg1122 (pK18 *oriV*$_{E.coli}$, *sacB*, *lacZ*α); synonym: pK18*mobsacB* NCR1/ *dapA-dapB-asd-lysA-ddh* /NCR2 | Blombach et al. (2009) |
| pK18*mobsacB*-P$_{tuf}$-*leuA*_B018 | Kan$^r$, pK18*mobsac*-lysOP7 derivative containing the *leuA* gene from B018 under control of the *tuf* promoter along with the 54 bp downstream region of *leuA* (XhoI/EcoRI) integrated into the intergenic region between cg1121 and cg1122 | This work |
| pK19*mobsacB*-GTG-*ilvE* | Kan$^r$, pK19*mobsacB* derivative for replacement of the ATG start codon of *ilvE* by GTG start codon | Haas (2013) |
| pJC1 | Kan$^r$; *E. coli*/*C. glutamicum* shuttle vector (*oriV*$_{E.coli}$, *oriV*$_{C.glutamicum}$) | Cremer et al. (1990) |
| pJC1-*leuCD* | Kan$^r$, pJC1 derivative containing genes *leuC* and *leuD* | Karin Krumbach (FZ Jülich, Germany) |
| pJC1-*leuB* | Kan$^r$, pJC1 derivative containing gene *leuB* | Karin Krumbach (FZ Jülich, Germany) |
| pJC1-*ilvE* | Kan$^r$, pJC1 derivative containing gene *ilvE* | Karin Krumbach (FZ Jülich, Germany) |
| pJC1-*brnFE* | Kan$^r$, pJC1 derivative containing genes *brnF* and *brnE* | Kennerknecht et al. (2002) |

[a] Kan$^r$; kanamycin resistance

## 2.2 DNA oligonucleotides

All DNA oligonucleotides used in this work, as well as their sequence and introduced recognition sites for restriction enzymes, are listed in Table 3. They were used for plasmid constructions, sequencing reactions, and confirmation of proper integration or deletion of DNA sequences in the chromosome of *C. glutamicum*. All DNA oligonucleotides were synthesized by Eurofins MWG Operon (Ebersfeld, Germany).

**Table 3.** DNA oligonucleotides used in this work.

| Name | DNA Sequence (5´- 3´)[a] | Restriction sites |
|---|---|---|
| **Oligonucleotides for plasmid constructions** | | |
| NdeI_leuA_B018_Fw | AAACATATGTCTCCTAACGATGCATTCATCTC CGCACCTGCCAAG | NdeI |
| leuA_ASAustausch_Fw | CGCGACAACGACCCACTGACCGCTTACGCCA AC | - |
| leuA_ASAustausch_Fw | AGCGGTCAGTGGGTCGTTGTCGCGGCCATC | - |
| EcoRI_leuA_B018_Rev | TTTGAATTCTTAAACGCCGCCAGCCAGGACTG CCTCGTGGTTGACGTCC | EcoRI |
| Sbf1_leuA_B018_intern_ Fw | AAAACCTGCAGGACCGTTGAATACTGCAACC AGCTGCGCG | SbfI |
| XbaI_leuA_int_down_ Rev | TTTTTCTAGAACCTCAATAGCTTCTTGGCGCTC AGCTTCGATTC | XbaI |
| SbfI_leuA_int_up_Fw | AAAACCTGCAGGGCGGAATACGTATTAAAGA AGGACAAAGCCCTC | SbfI |
| NdeI-MluI-SpeI-leuA_int_Fw | CATATGGGAACGCGTGGAACTAGTGCTTTAC GACGCCTCCCCCTAG | NdeI, MluI, SpeI |
| NdeI-MluI-SpeI-leuA_int_Rev | ACTAGTTCCACGCGTTCCCATATGGAGTTTTC TTTTAAAAAAGCTTTTCG | NdeI, MluI, SpeI |
| leuA_B018_amp_Fw | AAAACGCGTATGTCTCCTAACGATGCATTCAT CTCCGCACCTGCCAAG | MluI |

| Name | DNA Sequence (5´- 3´)[a] | Restriction sites |
|---|---|---|
| leuA_B018_amp_Rev | TTTACTAGTTTAAACGCCGCCAGCCAGGACTG CCTCGTGGTTGACGTCC | SpeI |
| NdeI_Peftu_Fw | GGGGCATATGCCACAGGGTAGCTGGTAGTTT GAAAATC | NdeI |
| MluI_Peftu_Rev | AAAAACGCGTTCCTCCTGGACTTCGTGGTGG CTAC | MluI |
| AvrII_Peftu_Fw | GGGGCCTAGGCCACAGGGTAGCTGGTAGTTT GAAAATC | AvrII |
| AvrII_leuA_Rev | AAACCTAGGCGGTGGCAAGAATTTTCAACATC GTGGAATTCTTGCCACCGGTTTGTAGAGGCT AGGGGG | AvrII |
| XhoI-Peftu_Fw | AAAACTCGAGCCACAGGGTAGCTGGTAG | XhoI |
| EcoRI-leuA_Rev | GGGGAATTCTTGCCACCGGTTTGTAGAG | EcoRI |
| SbfI_ilvN_up_Fw | AAAACCTGCAGGGCCCTTGGAGCAAAGGCTG | SbfI |
| XbaI_ilvN_down_Rev | GGGGTCTAGAGCAGTTCAATAGCCATGGGAG | XbaI |
| SOE_ilvN_Fw | GACGTAGACGATGACTTTTCCCGCGTATCAG GTATGTTCAC | - |
| SOE_ilvN_Rev | CGGGAAAAGTCATCGTCTACGTCCTGAACGA GTACGGAC | - |
| SbfI_AcT_Fw | AAAACCTGCAGGCTGCGTACGAGGGCAAGGA TCTTGTCAC | SbfI |
| EcoRI_AcT_Rev | GGGGGAATTCGCACCCAGAAAAAACCAATGC CAGGTAC | EcoRI |
| SOE_AcT_Fw | ACCCGTCACTCCACCATCTCCGCTGCTTCCG CAATGAGCTC | - |

| Name | DNA Sequence (5´- 3´)[a] | Restriction sites |
|---|---|---|
| SOE_AcT_Rev | GCTCATTGCGGAAGCAGCGGAGATGGTGGA GTGACGGGTATTTTGAG | - |
| **Oligonucleotides for sequencing/verification of integration or deletion of DNA sequences** | | |
| pEKEX-fw-2 | CGGCGTTTCACTTCTGAGTTCGGC | - |
| pEKEX-rv-2 | GATATGACCATGATTACGCCAAGC | - |
| rsp | CACAGGAAACAGCTATGACCATG | - |
| univ | CGCCAGGGTTTTCCCAGTCACGAC | - |
| leuA1+ | CCGAGACATGACAGCACTGGAACTGGG | - |
| leuA2+ | CGAGGTCGGTTTCCCTTCAGCTTCCC | - |
| leuA3+ | CACAATGACCGTGGCACCGGCGTTGGC | - |
| leuA4+ | CGGTCTGCAGATCCCTCGCTCCATGCAGG | - |
| leuA_int_c+ | CCACTCGATCGATGGATTCCAGTAC | - |
| leuA_int_c- | TCAATGGTGACCATGCGTGAAGTCG | - |
| ltbR_int_c+ | GAGCAGCAAATCTGGTGGTATAGC | - |
| ltbR_int_c- | AAATGTCACCTCCCGCCCAAAATC | - |
| ilvE_c+ | TTTCAAAACCACCGAGGGAGCGGGC | - |
| ilvE_c- | AGGCCGTCCATGAGGGCTTTGGTG | - |
| seqilvBNCD1+ | GAGCAAATATTGAATGGGTACGCC | - |
| seqilvBNCD2+ | GCTTCTCAACAGCCCACTCCCGCC | - |
| seqilvBNCD3+ | GATTACTGGTCGCCCTGGCCCTGTTC | - |
| seqilvBNCD4+ | TCCAATCGTGGGCGATGCCCGCGAAG | - |

| Name | DNA Sequence (5´- 3´)[a] | Restriction sites |
|---|---|---|
| seqilvBNCD5+ | CCTATTCTATGAAGGACGGTACTC | - |
| seqilvBNCD6+ | CTGCAAAGACCGAAACACACGGCATC | - |
| seqilvBNCD7+ | CGAGTTAAACTCCCACGATTTACAGTG | - |
| seqilvBNCD8+ | ACCAGGACCCAACCGGAACCG | - |
| seqilvBNCD9+ | GCCCTTTGGAATCGGTCCGAAAG | - |
| seqilvBNCD10+ | GTGCACACTTCTTGCCCAGGCCG | - |
| seqilvBNCD11+ | ACCGCGGTTGAGCTCGCCGAG | - |
| seqilvBNCD12+ | CTGCATCGCTTGCGGATGCGGAG | - |
| Delta-Q down kontr | GGTATTCATGTCCGTGCCAGG | - |
| Delta-Q up kontr | GCTCACCGATATTGCGGCG | - |
| pavtA-delfor | CTCCGGTCTGCTTTACGCAGG | - |
| pavtA-delrev | TGTGGGAACGGCCAGCCATGA | - |
| K_DiolR_fw | GCACGTTATGACCTGCAAACTC | - |
| K_DiolR_rev | TACGGTCTGGCTATCTACATCC | - |
| gltA upstream | ATCGTTAACGATCTGACCCAACAA | - |
| gltA downstream | TCGAGTGGGTTCAGCTGGTCC | - |
| d_AcT_c+ (cg3022) | GCTGAAGCAGCACCTGATCAATCAC | - |
| d_AcT_c- (cg3022) | TGAGAACAGCGCCGCGAGGGAATC | - |
| pK18_intcheck+ | CGGAATGATCTTGACCCTTGTTGGTG | - |
| pK18_intcheck- | ATCAAGCAGATCTCTGAGCTGCTGGC | - |

[a] Introduced recognition sites for restriction endonucleases are underlined.

## 2.3 Chemicals and growth media

### 2.3.1 Chemicals

All chemicals used in this work had a minimum quality of "*pro analysi*" (p.a.). All chemicals were purchased from Merck KGaA (Darmstadt, Germany), Fluka (Steinheim, Germany), Sigma-Aldrich (Taufkirchen, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Roche Diagnostics GmbH (Mannheim, Germany), and GE Healthcare (München, Germany). Components of complex growth media were purchased from Difco Laboratories (Detroit, USA).

### 2.3.2 Growth media

*C. glutamicum* was cultivated in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA), BHI medium with 9.1% (w/v) sorbitol (BHIS), CGIII complex medium (Menkel et al., 1989), or (modified) defined CGXII minimal medium (Keilhauer et al., 1993) with 4% (w/v) glucose as sole carbon source. Luria-Bertani (LB) medium (Bertani, 1951) was used for cultivation of *E. coli* DH5α.

BHI medium: 37 g/l BHI

BHIS medium: 37 g/l BHI, 91 g/l sorbitol

CGIII medium: 10 g/l peptone, 10 g/l yeast extract, 2.5 g/l NaCl, 20 g/l glucose; solution was adjusted to pH 7.5 before autoclaving; glucose was added after autoclaving

CGXII medium with 4% (w/v) glucose:
20 g/l $(NH)_4SO_4$, 5 g/l urea, 1 g/l $K_2HPO_4$, 1 g/l $KH_2PO_4$, 0.25 g/l $MgS0_4 \cdot 7H_2O$, 10 mg/l $CaCl_2$, 42 g/l 3-morpholinopropanesulfonic acid (MOPS), trace elements (10 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $MnSO_4 \cdot H_2O$, 1 mg/l $ZnSO_4 \cdot 7H_2O$, 0.2 mg/l $CuSO_4$, 0.02 mg/l $NiCl_2 \cdot 6H_2O$), 0.2 mg/l biotin, 30 mg/l protocatechuic acid, 40 g/l glucose; solution was adjusted to pH 7.0 with NaOH before autoclaving; trace elements, biotin, protocatechuic acid, and glucose were added after autoclaving

Modified CGXII medium with 4% (w/v) glucose:

20 g/l $(NH)_4SO_4$, 5 g/l urea, 1.2 g/l $K_2HPO_4$,
0.25 g/l $MgSO_4 \cdot 7H_2O$, 10 mg/l $CaCl_2$,
trace elements (10 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $MnSO_4 \cdot H_2O$,
1 mg/l $ZnSO_4 \cdot 7H_2O$, 0.2 mg/l $CuSO_4$, 0.02 mg/l $NiCl_2 \cdot 6H_2O$),
0.2 mg/l biotin, 30 mg/l protocatechuic acid; glucose, trace elements, biotin, and protocatechuic acid were added after autoclaving

LB medium: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl

For preparation of agar plates, 18 g/l agar was added to the medium before autoclaving.

Where appropriate, kanamycin (50 mg/ml stock solution, sterile-filtered) was added as selection marker to the medium to a final concentration of 25 µg/ml for *C. glutamicum* strains and 50 µg/ml for *E. coli* DH5α. To induce the expression of genes carried on pAN6 plasmids, 0.1 mM isopropyl β-D-thiogalactoside (IPTG) (1 M stock solution, sterile-filtered) was added to the medium. For 2-ketoisocaproate (KIC) production in shake flask experiments, pre- and main cultures were supplemented with L-isoleucine (and L-leucine) to a final concentration of 1 mM (100 mM stock solutions, sterile-filtered). For KIC production in bioreactors, 0.3 g/l L-isoleucine was added to the medium before autoclaving.

## 2.4 Cultivation of microorganisms

The *C. glutamicum* wild-type strain ATCC 13032 (Abe et al., 1967) and its recombinant derivatives were routinely cultivated aerobically at 30°C on agar plates, in test tubes, baffled Erlenmeyer flasks, or bioreactors. *E. coli* DH5α was cultivated aerobically at 37°C on agar plates and test tubes.

### 2.4.1 Maintenance of microorganisms

*C. glutamicum* cells were cultivated in test tubes with 5 ml BHI medium for 16 h at 30°C and 170 rpm. 800 µl of this culture were mixed with 800 µl sterile 28% (v/v) glycerol (in fresh BHI) and stored at -70°C. *E. coli* DH5α cells were cultivated in test tubes with 5 ml LB medium for 16 h at 37°C and 170 rpm. 600 µl of this culture were mixed with 300 µl sterile 85% (v/v) glycerol and stored at -70°C.

### 2.4.2 Cultivation in shake flasks

For bacterial growth as well as L-leucine and KIC production in shake flask cultivations, *C. glutamicum* strains were grown for 6-8 h in test tubes with 5 ml CGIII medium on a rotary shaker at 30°C and 170 rpm (first preculture) and subsequently inoculated into 50 ml CGXII medium with 4% (w/v) glucose in 500 ml baffled Erlenmeyer flasks. This second preculture was cultivated overnight on a rotary shaker at 30°C and 120 rpm and then used for the inoculation of the main culture to an $OD_{600}$ of 0.5 (if not stated otherwise). Medium and growth conditions for the main culture were the same as for the second preculture. During the cultivation, samples were taken for measuring $OD_{600}$ and then centrifuged at 16,000 x *g* for 5 min. The resulting cell-free supernatants were employed for the quantification of amino acids, glucose, and organic acids.

### 2.4.3 Cultivation in bioreactors

Batch and fed-batch cultivations of *C. glutamicum* strains for L-leucine and KIC production were performed in 1.4 l bioreactors of the Multifors multifermenter system from Infors GmbH (Einsbach, Germany). Each reactor was equipped with a suitable polarimetric oxygen electrode (Mettler-Toledo GmbH, Gießen, Germany) for measurement of the oxygen partial pressure (pO2), a suitable pH electrode (Mettler-Toledo), and an Exit Gas Analyzer (Infors) for exhaust gas analysis of $O_2$ and $CO_2$. Process parameters were controlled and monitored by IRIS software (Infors, Einsbach, Germany). Reactors were filled with modified CGXII minimal medium with 4% (w/v) glucose. Modified CGXII minimal medium was used because MOPS was not needed for buffering due to the automatic pH control (see below). The reduced phosphate amounts resulted in limitation of biomass in the feed phase. Cells were precultured in CGIII medium and CGXII minimal medium with 4% (w/v) glucose as it was described for the shake flask cultivations (section 2.4.2). The main culture in the bioreactor was inoculated with cells in the mid exponential phase ($OD_{600}$ = 10-15) to an $OD_{600}$ of 1 for starting the batch phase. The culture was aerated with a constant rate of 0.9 vvm (volume per volume per minute) with compressed air and kept at 30°C. The pH was kept constant at value 6.5, 7.2, or 8.0 by automatic addition of 3 M NaOH and 3 M HCl. A 25% (v/v) Antifoam 204/water suspension (Sigma-Aldrich, Steinheim, Germany) was added to the bioreactor to prevent foam formation when necessary. The stirrer speed was set to 500 rpm and was automatically increased up to 800 rpm to keep the dissolved oxygen saturation, represented by the $pO_2$ value, above 30%. When necessary, compressed air was mixed with pure oxygen to provide sufficient oxygen supply. The end of the batch phase was indicated by a raise of $pO_2$ to >80%, indicating a depletion of glucose. The feed phase started with the addition of feed solution (50% (w/v) glucose with or without 4% (w/v) ammonium

sulfate). Stirrer speed was manually adjusted to keep $pO_2$ >30% in the fed-batch mode. An automatic feeding program was conducted as follows: as soon as $pO_2$ raised above 80% again, feed solution was added to the bioreactor for 4 min (corresponding to approximately 15 ml feed solution). Samples were taken during the cultivation for measuring $OD_{600}$. Cell-free supernatants were obtained by centrifugation at 16,000 x *g* for 5 min and used for quantification of amino acids, glucose, and organic acids.

#### 2.4.4 Determination of bacterial growth

Bacterial growth was followed by measuring the optical density at 600 nm ($OD_{600}$) with an Ultrospec 3000 UV/visible spectrophotometer from Amersham Pharmacia Biotech (Freiburg, Germany). A water sample served as blank. To maintain linearity between absorbance and cell concentration, samples were diluted with water to gain absorbance values between 0.1 and 0.5.

### 2.5 Molecular biological work

Routine methods of molecular cloning like PCR, gel electrophoresis, DNA restriction, and ligation were carried out according to standard protocols (Sambrook and Russell, 2001) and manufacturer´s instructions.

#### 2.5.1 Preparation of plasmid DNA from *E. coli* cells

Plasmid DNA from *E. coli* DH5α cells was isolated using the GeneJET Plasmid Miniprep Kit from Thermo Scientific (Schwerte, Germany) according to the manufacturer's instructions. Cells used for plasmid preparation were grown in 5 ml LB medium in test tubes for 16 h at 37°C and 170 rpm on a rotary shaker.

#### 2.5.2 Preparation of genomic DNA from *C. glutamicum* cells

Genomic DNA from *C. glutamicum* cells was isolated using the DNAeasy Blood & Tissue Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions for application with gram-positive bacteria. Cells for this preparation were grown for 16 h in a test tube with 5 ml BHI medium at 30°C and 170 rpm on a rotary shaker.

### 2.5.3 Polymerase chain reaction

Polymerase chain reaction (PCR) (Mullis et al., 1986) was used for amplification of DNA fragments, for verification of cloning steps in *E. coli* DH5α cells, and for detection of desired integration or deletion mutants of *C. glutamicum* (colony-PCR) using a T3000 thermocycler from Biometra (Göttingen, Germany). For amplification of DNA fragments, the DNA polymerase Phusion Hot Start II from Thermo Scientific (Schwerte, Germany) was employed. DreamTaq DNA polymerase (Thermo Scientific) was used for verification of cloning steps and for colony-PCR. For the latter, a single colony was directly added to the PCR reaction mix. PCR setup (i.e. choice of reaction components, annealing temperature, elongation time) was done according to the manufacturer's instructions.

### 2.5.4 Purification of DNA fragments

For purification of DNA fragments after e.g. PCR or restriction digest, the MinElute PCR Purication Kit from Qiagen (Hilden, Germany) was used according to the manufacturer's instructions.

### 2.5.5 DNA agarose gel electrophoresis

For separation and sizing of DNA fragments, gel electrophoresis with 1% (w/v) agarose gels in TAE buffer (40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA)) was used. DNA samples were mixed with 6 x DNA Loading Dye from Thermo Scientific (Schwerte, Germany). Electrophoresis was carried out in Mini-Sub Cell GT electrophoresis chambers from Bio-Rad Laboratories GmbH (München, Germany) connected to a Power Pac 2000 station (Bio-Rad) at 90 V for 30-45 min. Afterwards, agarose gels were stained in an ethidium bromide solution (0.5 µg/ml). DNA bands were detected under UV light and documented using Quantum gel documentation system from Peqlab Biotechnologie GmbH (Erlangen, Germany). GeneRuler 1 kb DNA Ladder (Thermo Scientific) was employed for sizing of DNA fragments.

### 2.5.6 Extraction of DNA fragments from agarose gels

The MinElute Gel Extraction Kit from Qiagen (Hilden, Germany) was used for isolation of DNA fragments from agarose gels according to the manufacturer's instructions.

### 2.5.7 Determination of nucleic acid concentration

Concentration of DNA and RNA samples was determined by measuring the absorbance at 260 nm ($A_{260nm}$) using a NanoDrop ND-1000 spectrophotometer from Peqlab Biotechnologie GmbH (Erlangen, Germany). Purity of nucleic acid preparations was displayed by the ratio of the absorbance value at 260 nm ($A_{260nm}$) and the absorbance value at 280 nm ($A_{280nm}$). This ratio should be 1.8-2.0 for pure DNA or RNA. $A_{260nm}$ = 1 is equal to 50 ng/µl double stranded DNA or 40 ng/µl RNA, respectively.

### 2.5.8 DNA sequencing

DNA sequencing was provided by Eurofins MWG Operon (Ebersfeld, Germany) and used for verification of constructed plasmids as well as chromosomal deletions or integrations in engineered strains. Samples were prepared according to the provider's instructions and sequencing results were analyzed using the software CloneManager 9 Professional Edition (Scientific & Educational Software, Cary, USA).

### 2.5.9 Restriction digest and modification of DNA

FastDigest restriction endonucleases from Thermo Scientific (Schwerte, Germany) were used for restriction digest of DNA templates according to the manufacturer's instructions. Reactions were carried out for 0.5-2 h at 37°C. To prevent religation of plasmid DNA, 1 µl Shrimp Alkaline Phosphatase (Thermo Scientific) was added to the respective samples.

### 2.5.10 Ligation of DNA

For ligation of digested DNA fragments, the Rapid DNA Ligation Kit from Thermo Scientific (Schwerte, Germany) was used according to the manufacturer's instructions. Ligation reaction was carried out for 30-60 min at RT.

### 2.5.11 Preparation and transformation of competent *C. glutamicum* cells

For preparation of competent cells, *C. glutamicum* strains were inoculated into 5 ml BHIS medium in a test tube and grown overnight on a rotary shaker at 30°C and 170 rpm. 100 ml BHIS medium in a 1000 ml baffled Erlenmeyer flask were inoculated with this preculture and grown on a rotary shaker at 30°C and 120 rpm to an $OD_{600}$ of approximately 1.75. Cells were harvested by centrifugation (25 min; 4,000 x *g*; 4°C) and washed three times with cold sterile 10% (v/v) glycerol via centrifugation steps (15 min; 4,000 x *g*; 4°C). Finally, cells were

resuspended in 1 ml sterile 10% (v/v) glycerol. Cells were frozen in liquid nitrogen as 150 µl aliquots and stored at -70°C.

Transformation of competent *C. glutamicum* cells was done by electroporation and subsequent heat-shock (van der Rest et al., 1999). 150 µl competent cells were mixed with 0.2-1 µg of DNA in a precooled electroporation cuvette (Typ Gene Pulser Cuvette, 0.1 cm, Bio-Rad Laboratories GmbH, München, Germany) and coated with 800 µl cold sterile 10% (v/v) glycerol. Electroporation (settings: 2.5 kV, 200 Ω, 25 µF) was carried out with the Gene Pulser Xcell device from Bio-Rad Laboratories GmbH (München, Germany). Afterwards, cells were incubated for 6 min in 4 ml BHIS medium at 46°C (heat shock), regenerated for at least 1 h at 30°C and 170 rpm on a rotary shaker, and eventually plated on BHIS agar with the respective selection marker (kanamycin).

### 2.5.12 Preparation and transformation of competent *E. coli* cells

For preparation of competent cells (Hanahan, 1983), *E. coli* DH5α cells were inoculated into 5 ml LB medium in a test tube and grown overnight on a rotary shaker at 37°C and 170 rpm. 100 µl of this preculture were used for inoculation of 70 ml LB medium in a 500 ml baffled Erlenmeyer flask. Cells were grown to an $OD_{600}$ of approximately 0.5 at 37°C and 120 rpm, chilled in ice-cold water, and then harvested by centrifugation (10 min; 4,000 x *g*; 4°C). The cell pellet was resuspended in 15 ml sterile 70 mM $CaCl_2$/20 mM $MgSO_4$ solution, incubated for 30 min on ice, and then harvested by centrifugation (10 min; 4,000 x *g*; 4°C). After resuspension in 5 ml sterile 70 mM $CaCl_2$/20 mM $MgSO_4$ solution, cells were incubated on ice for 30 min. Finally, 1.25 ml sterile 85% (v/v) glycerol was added. Competent cells were frozen in liquid nitrogen as 200 µl aliquots and stored at -70°C.

Competent *E. coli* DH5α cells were transformed with 10-100 ng of plasmid DNA via heat-shock: Cells were incubated for 90 sec at 42°C and then regenerated in 800 µl LB medium for 1 h at 37°C and 170 rpm on a rotary shaker. Eventually, cells were plated on LB agar with the respective selection marker (kanamycin).

### 2.5.13 Integration and deletion of genes using the pK19*mobsacB* system

Marker-free integrations or in-frame deletions of DNA sequences in *C. glutamicum* strains were done via two-step homologous recombination based on vector pK19*mobsacB* (Schäfer et al., 1994) and a method described by Niebisch and Bott (2001): DNA sequences to be integrated or deleted were cloned together with their >500 bp up- and downstream regions into vector pK19*mobsacB*. After transformation of the respective *C. glutamicum* strain, clones with integrated vector were selected using BHIS agar plates with 25 µg/ml kanamycin. Since pK19*mobsacB* is a non-replicative plasmid, appearance of kanamycin-resistant cells

depends on integration of the plasmid into the genome via homologous recombination. For excision of the vector, clones were incubated in 5 ml BHI medium without kanamycin in test tubes at 30°C and 170 rpm on a rotary shaker for 3-5 h and plated on BHI agar plates with 10% (w/v) sucrose. The vector pK19*mobsacB* carries the gene *sacB*. This gene codes for the enzyme levansucrase that catalyzes sucrose hydrolysis and synthesis of levan, which is lethal to *C. glutamicum* (Jäger et al., 1992). Using plates with sucrose, only clones which had had excised the vector were selected. Finally, clones which showed both sucrose-resistance and kanamycin-sensitivity were checked via colony-PCR and DNA sequencing for proper gene integration or deletion.

### 2.5.14 Plasmid construction

For the construction of pAN6-*leuA*_wt and pAN6-*leuA*_B018, gene *leuA* encoding for 2-isopropylmalate synthase was amplified using the oligonucleotide pair NdeI_leuA_B018_Fw/EcoRI_leuA_B018_Rev from genomic DNA of *C. glutamicum* wild type and B018, respectively. The resulting DNA fragments were each digested with the restriction enzymes NdeI and EcoRI and separately ligated into the plasmid pAN6 cut with the same enzymes.

Overlap-extension PCR was applied for the construction of plasmid pAN6-*leuA*_mod: Two DNA fragments (276 bp and 1614 bp, respectively) were amplified from genomic DNA of *C. glutamicum* wild type via the oligonucleotide pairs NdeI_leuA_B018_Fw/ leuA_ASAustausch_Rev and leuA_ASAustausch_Fw/EcoRI_leuA_B018_Rev, together covering gene *leuA*. The two fragments were fused via a 24 bp overlap-region in a PCR reaction to yield a *leuA* gene with mutations leading to amino acid exchanges G530D, G532D, and A535T. This gene was amplified using the oligonucleotides NdeI_leuA_B018_Fw and EcoRI_leuA_B018_Rev, digested with the restriction endonucleases NdeI/EcoRI and finally ligated into the equally digested plasmid pAN6.

To obtain the vector pK19*mobsacB*-*leuA*_B018_BS, a 1378 bp PCR fragment was generated from genomic DNA of B018 using the oligonucleotides Sbf1_leuA_B018_intern_Fw and XbaI_leuA_int_down_Rev. It covered the partial sequence of *leuA* from B018 coding for the amino acid exchanges R529H and G532D in comparison to the wild-type sequence along with its upstream and downstream regions (approximately 560 bp and 810 bp, respectively). The fragment was cut with the restriction endonucleases SbfI and XbaI and ligated into the vector pk19*mobsacB* that had been digested with the same enzymes.

The vector pK19*mobsacB*-Δ*leuA* was constructed via overlap-extension PCR: The region upstream (approximately 490 bp) of the 180 bp upstream region of *leuA* as well as the downstream region (approximately 550 bp) of *leuA* were amplified via the oligonucleotide

pairs SbfI_leuA_int_up_Fw/NdeI-MluI-SpeI-leuA_int_Rev and NdeI-MluI-SpeI-leuA_int_Fw/ XbaI_ leuA_int_down_Rev, respectively, from genomic DNA of *C. glutamicum* wild type. The amplified DNA fragments were fused in an overlap-extension PCR resulting in a 1060 bp PCR product that was amplified with the oligonucleotides SbfI_leuA_int_up_Fw and XbaI_leuA_int_down_Rev. This fragment was digested with the restriction enzymes SbfI/ XbaI and ligated into the equally digested vector pK19*mobsacB*.

The plasmid pK19*mobsacB*-$P_{tuf}$-*leuA*_B018 was constructed as follows: The gene *leuA* from B018 (*leuA*_B018) was amplified via the oligonucleotides leuA_B018_amp_Fw and leuA_B018_amp_Rev; the sequence of the *tuf* promoter ($P_{tuf}$, 178 bp upstream of the coding sequence of the *tuf* gene) (Becker et al., 2005) was amplified using the oligonucleotide pair NdeI_Peftu_Fw/MluI_Peftu_Rev. First, the *leuA*_B018 PCR fragment was cut with the restriction enzymes MluI and SpeI and ligated into pK19*mobsacB*-Δ*leuA*. In a second step, to finally obtain plasmid pK19*mobsacB*-$P_{tuf}$-*leuA*_B018, the resulting plasmid was cut with the endonucleases NdeI and MluI and ligated with the $P_{tuf}$ DNA fragment digested with the same enzymes.

For the construction of vector pK19*mobsacB*-Δ*ltbR*::$P_{tuf}$-*leuA*_B018, the *leuA* gene from B018 under control of the *tuf* promoter and, to cover the transcription terminator sequence of this gene, the region approximately 70 bp upstream of *leuA* were amplified by use of oligonucleotides AvrII_Peftu_Fw and AvrII_leuA_Rev. The program Mfold (Zuker, 2003) was used to predict the transcription terminator sequence of *leuA* (http://mfold.rna.albany.edu/?q=mfold). The resulting PCR fragment was digested with AvrII and ligated into pK19*mobsacB*-Δ*ltbR* which had been digested in the same way.

The plasmid pK18*mobsacB*-$P_{tuf}$-*leuA*_B018 was constructed similarly by amplifying the coding sequence for *tuf* promoter and *leuA* from B018 along with its 54 bp downstream region to cover the transcription terminator sequence from pK19*mobsacB*-$P_{tuf}$-*leuA*_B018 via the oligonucleotides XhoI-Peftu_Fw and EcoRI-leuA_Rev. The DNA fragment was digested with the restriction enzymes XhoI and EcoRI and ligated with the 7.2 kb plasmid backbone originating from plasmid pK18*mobsacB*-lysOP7 digested with the restriction enzymes XhoI and EcoRI.

Overlap-extension PCR was applied for the construction of vector pK19*mobsacB*-*ilvN*_fbr: The upstream region (approximately 620 bp) and the downstream region (approximately 660 bp) of the partial sequence of *ilvN* coding for the amino acids G20, I21, and I22 was amplified via the oligonucleotide pairs SbfI_ilvN_up_Fw/SOE_ilvN_Rev and SOE_ilvN_Fw/XbaI_ilvN_down_Rev, respectively, from genomic DNA of *C. glutamicum* wild type. The amplified DNA fragments were fused in a PCR reaction via a 23 bp overlap-region that introduced mutations into *ilvN* leading to amino acid exchanges G20D, I21D, and I22F. This 1279 bp fusion product was amplified using oligonucleotides SbfI_ilvN_up_Fw and

XbaI_ilvN_down_Rev, digested with the restriction endonucleases SbfI and XbaI, and finally ligated into the equally digested vector pK19*mobsacB*.

The vector pK19*mobsacB*-Δcg3022 was constructed via overlap-extension PCR: The regions upstream (approximately 570 bp) and downstream (approximately 510 bp) of cg3022 were amplified via the oligonucleotide pairs SbfI_AcT_Fw/SOE_AcT_Rev and SOE_AcT_Fw/EcoRI_AcT_Rev, respectively, from genomic DNA of *C. glutamicum* wild type. The amplified DNA fragments were fused and the resulting 1172 bp fragment amplified via the oligonucleotides SbfI_AcT_Fw and EcoRI_AcT_Rev. This fragment was digested with the restriction enzymes SbfI/EcoRI and ligated into the equally digested vector pK19*mobsacB*.

### 2.5.15 Preparation of RNA

For preparation of total RNA of *C. glutamicum* strains, cells were cultivated in 50 ml CGXII minimal medium with 4% (w/v) glucose to an $OD_{600}$ of 5. An aliquot of 25 ml was centrifuged (3 min; 4,000 x *g*; 4°C) with 25 g ice. The cell pellet was frozen in liquid nitrogen and stored at -70°C. Mechanical cell disruption was performed via Silamat S5 from Ivoclar Vivadent GmbH (Ellwangen, Germany) using 400 mg silica beads (diameter 0.1 mm) for 4 x 20 sec. The isolation and purification of RNA was done using the RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Integrity of RNA was checked via RNA agarose electrophoresis before further use and isolated RNA (100-150 µg) was stored at -20°C.

### 2.5.16 RNA agarose gel electrophoresis

For separation of RNA fragments, denaturing gel electrophoresis with 1.5% (w/v) agarose gels in MOPS buffer (50 mM MOPS, 1 mM EDTA, 1.5% (v/v) formaldehyde) was used. Before electrophoresis, RNA samples were mixed with the fourfold amount of RNA loading dye (50 mM MOPS, 50% (v/v), formamide, 7% (v/v) formaldehyde, 15% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 0.5% (w/v) ethidium bromide), denatured for 2 min at 65°C, and then chilled on ice for 5 min. Electrophoresis was carried out in Mini-Sub Cell GT electrophoresis chambers from Bio-Rad Laboratories GmbH (München, Germany) connected to a Power Pac 2000 station (Bio-Rad) at 75 V for 45 min. RNA bands were detected under UV light and documented using Quantum gel documentation system from Peqlab Biotechnologie GmbH (Erlangen, Germany).

### 2.5.17 Synthesis and labeling of cDNA

Isolated total RNA samples were converted into fluorescently labeled cDNA (Polen et al., 2007). 30 µg of RNA were mixed with 500 ng Random Primers from Invitrogen (Karlsruhe, Germany), filled up with RNase-free water to 15 µl, incubated for 10 min at 65°C, and then chilled on ice for 2 min. Samples were either mixed with 3 µl of 1 mM green-fluorescing nucleotide analoge FluoroLink Cy3-dUTP or red-fluorescing FluoroLink Cy5-dUTP from GE Healthcare (München, Germany). Then, 11.6 µl of a reverse transcriptase mastermix were added.

Reverse transcriptase mastermix: 6 µl 0.1 M dithiotreitol
12 µl 5 x 1st Strand Buffer (Invitrogen)
1.2 µl dNTP-Mix (Invitrogen):
25 mM dATP, 25 mM dCTP,
25 mM dGTP, 10 mM dTTP
4 µl Superscript II Reverse Transcriptase (Invitrogen)

Samples were incubated for 10 min at RT and then for 110 min at 42°C. Reaction was stopped by adding 10 µl 0.1 M NaOH at 70°C to hydrolyze RNA. 10 µl 0.1 M HCl were used for neutralization.

For sample purification, Microcon YM-30 columns from Millipore (Schwalbach, Germany) were employed. 50 µl of sample were mixed with 450 µl bidist. $H_2O$ in the column and the volume was reduced to 20 µl by centrifugation (10 min; 16,000 x *g*; RT). The column was refilled to 500 µl and the volume was reduced to 20 µl again. Cy3- and Cy5-marked samples to be compared in DNA microarray analysis were pooled and refilled to 500 µl with bidist. $H_2O$ in a Microcon column. Finally, the volume was reduced to < 44 µl by centrifugation and samples were directly used for hybridization on DNA microarrays.

### 2.5.18 DNA microarray analysis

Custom-made 4 x 44K 60mer DNA microarrays for genome-wide gene expression analysis were obtained from Agilent Technologies (Waldbronn, Germany) and were designed using Agilent's eArray platform (https://earray.chem.agilent.com/earray). The array design included oligonucleotides for annotated protein-coding genes and structural RNA genes of the genomes from *C. glutamicum* and three other bacterial species (*E. coli*, *Gluconobacter oxydans*, and *Bacillus subtilis*). For *C. glutamicum*, the genome annotation by Kalinowski et al. (2003) listing 3057 protein-coding genes and 80 structural RNA genes (tRNAs and rRNAs) was used (accession number NC_006958). The custom array design

comprised each of these genes, represented by one to three oligonucleotides, and Agilent's control spots.

For comparison of global gene expression of selected *C. glutamicum* strains, pooled labeled cDNA was hybridized at 65°C for 17 h on the 4 x 44K arrays using Agilent's gene expression hybridization kit, Agilent's hybridization chamber, and Agilent's hybridization oven according to the manufacturer's instructions. After hybridization, the arrays were washed using Agilent's wash buffer kit according to the manufacturer's instructions. Subsequently the fluorescence of DNA microarrays was determined at 532 nm (Cy3-dUTP) and 635 nm (Cy5-dUTP) at 5 µm resolution with a GenePix 4000B laser scanner and GenePix Pro 6.0 software from Molecular Devices (Sunnyvale, USA). Raw data files of fluorescence images were saved in TIFF format (GenePix Pro 6.0) followed by quantitative image analysis using GenePix image analysis software and Agilent's gene array list (GAL) file. Results were saved as GPR-file (GenePix Pro 6.0) and processed using the BioConductor R-packages limma and marray (http://www.bioconductor.org) to achieve background correction of spot intensities, ratio calculation/normalization, and diagnostic-plot generation for array quality control. For analysis, processed and loess-normalized data along with detailed information about the experiments according to the MIAME standard (Brazma, 2009) were saved in the in-house DNA microarray database (Polen and Wendisch, 2004). Analysis of differentially expressed genes was carried out using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, USA) by filtering processed Cy5/Cy3 ratios reflecting the relative mRNA level using the following criteria: (i) Flags ≥ 0; (ii) signal/noise of Cy5 (F635Median/B635Median) or Cy3 (F532Median/B532Median) ≥ 3. For calculation of *p*-values, paired Student's *t*-test was used comparing the relative RNA levels of a gene in the replicates to the relative RNA levels of all other genes in the replicates.

## 2.6 Quantification of metabolites

### 2.6.1 Quantification of amino acids

Reversed phase high-pressure liquid chromatography (RP-HPLC) with automatic *o*-phthaldialdehyde precolumn derivatization (Jones & Gilligan 1983) was used for amino acid quantification in culture supernatants using a 1290 Infinity System from Agilent Technologies (Waldbronn, Germany) equipped with a ZORBAX Eclipse AAA column (4.6 x 75 mm 3.5-Micron), a ZORBAX Analytical Guard column (4.6 x 12.5 mm 5-Micron), and an Agilent 1260 fluorescence detector. A gradient of 10 mM $Na_2HPO_4$/10 mM $Na_2B_4O_7$ (pH 8.2) buffer with increasing concentrations of methanol was used as eluent. HPLC-samples were separated at 40°C at a flow rate of 2 ml $min^{-1}$ for 11.5 min. The detection of the

fluorescent isoindole derivatives was performed using an excitation wavelength of 230 nm and an emission wavelength of 450 nm. The concentration of the amino acids was determined by comparison with defined external standards of the respective amino acids. The program Chemstation (Agilent) was used for data evaluation. HPLC-samples were diluted 1:100-1:1000.

### 2.6.2 Quantification of organic acids and glucose

The detection and quantification of organic acids and glucose in culture supernatants was performed using an Agilent 1100 liquid chromatography system (Agilent Technologies, Waldbronn, Germany) equipped with an organic acid column (300 x 8 mm polystyrol-divinylbenzol resin) from CS Chromatographie Service GmbH (Langerwehe, Germany) and a suitable guard cartridge (40 x 8 mm). Organic acids and glucose were separated by isocratic elution at 40°C with 100 mM sulfuric acid for 38 min at a flow rate of 0.4 ml $min^{-1}$. Samples were diluted 1:4. Separation of keto acids (dilution 1:10) in particular was performed using isocratic elution with 10 mM sulfuric acid for 15 min at 70°C and a flow rate of 1.1 ml $min^{-1}$. Organic acids were detected via an Agilent 1100 diode array detector at 215 nm; glucose was detected via an Agilent 1100 refractive index detector. Quantification was performed with the program Chemstation (Agilent) using external standards of glucose and the respective organic acids.

### 2.6.3 Calculation of glucose uptake rate

Glucose uptake rates were determined as described by Frunzke et al. (2008):

$$\left(\frac{\mathrm{S}}{\mathrm{M}}\right) \times \mu \left[\left(\frac{\mathrm{mmol} \times \mathrm{l}^{-1} \times \mathrm{OD}_{600}{}^{-1}}{\mathrm{g\ CDW} \times \mathrm{l}^{-1} \times \mathrm{OD}_{600}{}^{-1}}\right) \times \mathrm{h}^{-1}\right] = \left[\frac{\mathrm{mmol}}{\mathrm{g\ CDW} \times \mathrm{h}}\right]$$

S represents the slope of a plot of the substrate concentration in the medium versus the $OD_{600}$ (mmol $l^{-1}$ $OD_{600}{}^{-1}$), M is the correlation between cell dry weight (CDW) and $OD_{600}$ (g CDW $l^{-1}$ $OD_{600}{}^{-1}$) and $\mu$ is the growth rate ($h^{-1}$).
Cell dry weight was calculated according to Kabus et al. (2007): An $OD_{600}$ of 1 corresponds to 0.25 g CDW $l^{-1}$.

## 2.7 Protein biochemical methods

### 2.7.1 Preparation of crude cell extract

Crude cell extracts were prepared for the determination of 2-isopropylmalate synthase (IPMS) and 3-isopropylmalate dehydratase (IPMD) activity. Cells were grown in CGXII minimal medium with 4% (w/v) glucose to an $OD_{600}$ of 5, harvested by centrifugation (10 min; 4,000 x *g;* 4°C), and then resuspended in 1 ml 50 mM Tris-HCl buffer (pH 7.5) with 200 mM potassium glutamate for the IPMS assay or in 200 mM potassium phosphate buffer (pH 7.0) for the IPMD assay. Cell suspensions were disrupted by sonication for 5 min on ice using a Branson Sonifier W-250 (Branson Sonic Power, Danbury, USA) and cell debris was removed by centrifugation (5 min; 16,000 x *g*; 4°C). The resulting crude extracts with protein concentrations of 2-5 mg $ml^{-1}$ were finally used for the respective enzyme assays.

### 2.7.2 Determination of protein concentration

Protein concentrations of crude extracts were determined by a modified Lowry assay as described by Bensadoun and Weinstein (1976): 1 ml crude extract (diluted 1:100-1:200) was mixed with 50 µl 0.3% (w/v) sodium deoxycholate and incubated for 15 min at RT. After mixing with 200 µl 36% (w/v) trichloroacetate, the solution was centrifuged (10 min; 16,000 x *g*; RT) and the supernatant was discarded. 1 ml Lowry reagent (0.01% (w/v) $CuSO_4 \cdot 5H_2O$, 0.02% (w/v) sodium potassium tartrate, 0.02% (w/v) $Na_2CO_3$ in 0.1 M NaOH) was added to the protein pellet, mixed, and incubated for 30 min at RT in the dark after adding 100 µl diluted Folin's reagent (1:2 in bidist. $H_2O$). Finally, absorbance was measured at 660 nm with an Ultrospec 3000 UV/visible spectrophotometer from Amersham Pharmacia Biotech (Freiburg, Germany). A mix of Lowry solution and Folin's reagent was used as blank. The protein concentrations were determined by comparison with a defined standard of 0-50 µg bovine serum albumin which was treated like the samples.

### 2.7.3 Enzyme assay for 2-isopropylmalate synthase

2-Isopropylmalate synthase (IPMS) activity, $K_M$ values for 2-ketoisovalerate (KIV) and acetyl-CoA, as well as $K_i$ value for L-leucine were determined at 30°C using a continuous spectrophotometric assay measuring coenzyme A formation with Ellmann's reagent (Kohlhaw, 1988a). The assay solution contained 500 µl 50 mM Tris-HCl buffer (pH 7.5) with 400 mM potassium glutamate, 100 µl 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (1 mM in 50 mM Tris-HCl, pH 7.5), 50 µl acetyl-CoA solution (3 mM in 50 mM Tris-HCl, pH 7.5), 295 µl bidist. $H_2O$, and 50 µl crude extract (diluted 1:1-1:5). The reaction was started

by adding 5 µl KIV solution (40 mM in 50 mM Tris-HCl, pH 7.5). Liberated CoA was measured at 412 nm with an Ultrospec 3000 UV/visible spectrophotometer from Amersham Pharmacia Biotech (Freiburg, Germany). Enzyme activities were calculated with SWIFT II software from Biochrom Ltd. (Cambridge, UK) using an extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$ for the 5-thio-2-nitrobenzoate dianion.

### 2.7.4 Enzyme assay for 3-isopropylmalate dehydratase

3-Isopropylmalate dehydratase (IPMD) activity was determined at 30°C using a continuous spectrophotometric assay measuring formation of the reaction intermediate 2-isopropylmaleate (Kohlhaw, 1988b). 50 µl crude extract (diluted 1:1-1:100) was mixed with 400 µl 200 mM potassium phosphate buffer (pH 7.0) and 510 µl bidist. $H_2O$. The reaction was started by adding 40 µl 3-isopropylmalate solution (40 mM in bidist. $H_2O$). The increase of absorbance at 235 nm was measured with an Ultrospec 3000 UV/visible spectrophotometer from Amersham Pharmacia Biotech (Freiburg, Germany). Enzyme activities were calculated with SWIFT II software from Biochrom Ltd. (Cambridge, UK) using an extinction coefficient of 4,530 $M^{-1}$ $cm^{-1}$ for 2-isopropylmaleate.

# 3 Results

## 3.1 The role of *leuA*-encoded 2-isopropylmalate synthase for increased L-leucine production

### 3.1.1 Analysis of the L-leucine producer B018

As a starting-point for this work, a weak L-leucine accumulating *C. glutamicum* strain named B018 was obtained from Amino GmbH (Frellstedt, Germany). This strain had been generated by the classical approach of random mutagenesis and subsequent screening for production. In shake flask cultivations with CGXII minimal medium and 4% (w/v) glucose, B018 accumulated 7.8 ± 2.0 mM L-leucine. Strain B018 had no auxotrophies and grew in the defined mimimal medium CGXII, but showed a significantly reduced growth rate ($\mu$) of 0.23 ± 0.01 $h^{-1}$ in comparison to the wild-type ATCC 13032 ($\mu$ = 0.44 ± 0.01 $h^{-1}$). As a first step to elucidate the reason for the ability of B018 to produce L-leucine, genes *ilvBNCD*, that code for enzymes catalyzing the formation of the L-leucine precursor 2-ketoisovalerate (KIV), and *leuA* encoding 2-isopropylmalate synthase (IPMS), were sequenced. The latter enzyme catalyzes the first step specific for L-leucine biosynthesis, the conversion of KIV and acetyl-CoA to 2-isopropylmalate and CoA, and is feedback-inhibited by L-leucine (Pátek et al., 1994). There were no mutations found in *ilvBNCD*, but the sequence of *leuA* from B018 showed differences with respect to the sequence of the wild-type ATCC 13032 (Kalinowski et al., 2003): Two nucleotide exchanges (G1586A, G1595A) were identified, leading to amino acid exchanges R529H and G532D (Table 4). Subsequently, *leuA* from B018 was characterized to prove whether this allele is responsible for L-leucine accumulation.

### 3.1.2 Characterization of the *leuA*-encoded IPMS from wild-type ATCC 13032 and B018

The *leuA* genes from *C. glutamicum* wild type and strain B018 (*leuA*_wt and *leuA*_B018, respectively) were cloned into the expression vector pAN6, resulting in plasmids pAN6-*leuA*_wt and pAN6-*leuA*_B018. These plasmids were transferred into *C. glutamicum* wild type and the IPMS activity of crude extracts was determined. Both strains showed comparable specific IPMS activities of 0.59-0.63 µmol $min^{-1}$ $mg_{protein}^{-1}$, which were 10- to 20-fold higher than the activities of the wild type and strain B018 (Table 5). Remarkably, the affinities of the mutated IPMS towards its substrates KIV and acetyl-CoA were comparable to those of the wild-type enzyme, but the feedback-inhibition by L-leucine was abolished (Table 6). The activity of wild-type IPMS was 50% inhibited by 0.4 mM L-leucine, whereas IPMS activity from strain B018 was not inhibited even by 40 mM L-leucine.

Next, L-leucine accumulation was assayed: The strain carrying pAN6-*leuA*_wt accumulated 0.8 ± 0.1 mM L-leucine, whereas the strain with pAN6-*leuA*_B018 accumulated 40.7 ± 0.4 mM L-leucine in the culture supernatant. These data disclose the significance of feedback-resistant IPMS for L-leucine overproduction. In order to test the influence of feedback-resistant IPMS in single copy, the *leuA* mutations R529H and G532D were introduced into the wild-type genome. The resulting strain MV-Leu7 accumulated 6.9 ± 0.5 mM L-leucine (Figure 4), indicating that also increased expression of *leuA*_B018 is necessary to obtain high L-leucine concentrations.

### 3.1.3 Construction of further feedback-resistant IPMS

IPMS of *C. glutamicum* shares 61% amino acid sequence identity with the homologous enzyme of *Mycobacterium tuberculosis* and the crystal structure of the mycobacterial protein revealed that the amino acid residues G531, G533, and A536 form an L-leucine-binding pocket (Koon et al., 2004). The corresponding residues in IPMS from *C. glutamicum* are G530, G532, and A535. Since the amino acid exchanges found in IPMS of B018 (R529H and G532D) are located in this region, it was assumed that the feedback-resistance of this variant is attributed to an impairment of its L-leucine-binding pocket. Another modified IPMS variant (IPMS_mod) was constructed carrying the amino acid exchanges G530D, G532D, and A535T to completely disturb the binding pocket (Table 4). The respective *leuA*_mod gene was cloned into the expression vector pAN6, resulting in pAN6-*leuA*_mod that was transferred into *C. glutamicum* wild type. IPMS_mod indeed showed no feedback-inhibition as IPMS_B018 (Table 6), but exhibited a decreased specific activity and lower L-leucine accumulation in comparison to IPMS from B018 when performing plasmid-based overexpression in shake flask cultivations (Table 5). Therefore, IPMS_B018 was used for further studies.

**Table 4. Differences in DNA and amino acid sequences of wild-type IPMS (IPMS_wt), IPMS from strain B018 (IPMS_B018), and constructed modified IPMS (IPMS_mod)[a,b].**

| Amino acid position | 529 | 530 | 531 | 532 | 533 | 534 | 535 |
|---|---|---|---|---|---|---|---|
| IPMS_wt: | | | | | | | |
| DNA codon | CGC | GGC | AAC | GGC | CCA | CTG | GCC |
| Amino acid | R | G | N | G | P | L | A |
| IPMS_B018: | | | | | | | |
| DNA codon | C**A**C | GGC | AAC | G**A**C | CCA | CTG | GCC |
| Amino acid | **H** | G | N | **D** | P | L | A |
| IPMS_mod: | | | | | | | |
| DNA codon | CGC | G**A**C | AAC | G**A**C | CCA | CTG | **A**CC |
| Amino acid | R | **D** | N | **D** | P | L | **T** |

[a] The partial IPMS amino acid sequence from position 529 to 535 and its respective DNA codons are shown.
[b] Mutated nucleotides and amino acids with respect to wild-type IPMS are in boldface.

**Table 5. Specific 2-isopropylmalate synthase activities and L-leucine accumulation of *C. glutamicum* wild type and different plasmid-based L-leucine producers in shake flask cultivations in CGXII minimal medium with 4% (w/v) glucose[a,b].**

| Strain | Specific IPMS activity ($\mu mol\ min^{-1}\ mg_{protein}^{-1}$) | L-leucine titer[d] (mM) |
|---|---|---|
| *C. glutamicum* wild type | 0.06 ± 0.01 | 0 |
| *C. glutamicum* pAN6-*leuA*_wt | 0.59 ± 0.02 | 0.8 ± 0.1 |
| B018 | 0.03 ± 0.01[c] | 7.8 ± 2.0 |
| *C. glutamicum* pAN6-*leuA*_B018 | 0.63 ± 0.04 | 40.7 ± 0.4 |
| *C. glutamicum* pAN6-*leuA*_mod | 0.21 ± 0.01 | 26.1 ± 0.2 |

[a] Strains carrying plasmids were induced with 0.1 mM IPTG.
[b] All data represent mean values with standard deviations obtained from at least three independent measurements with different crude extracts.
[c] Reduction of IPMS activity in B018 compared to *C. glutamicum* wild type is attributed to attenuation of *leuA* due to elevated L-leucine concentrations (Pátek et al., 1994).
[d] L-Leucine titers represent maximal values reached after 32 h at the latest.

**Table 6. Catalytic characteristics ($K_M$ and $K_i$ values) of 2-isopropylmalate synthase from *C. glutamicum* wild type (IPMS_wt) and strain B018 (IPMS_B018), as well as of constructed IPMS_mod[a,b].**

| | IPMS_wt | IPMS_B018 | IPMS_mod |
|---|---|---|---|
| $K_M$ 2-ketoisovalerate (µM) | 6.4 ± 0.1 | 10.0 ± 0.2 | 12.0 ± 0.2 |
| $K_M$ acetyl-CoA (µM) | 9.6 ± 0.4 | 9.7 ± 0.4 | 12.8 ± 0.7 |
| $K_i$ L-leucine (mM) | 0.4 ± 0.1 | >40 (feedback-resistant)[c] | >40 (feedback-resistant)[c] |

[a] Cells were cultivated in shake flasks using CGXII minimal medium with 4% (w/v) glucose.
[b] All data given are mean values with standard deviations obtained from at least three independent measurements with different crude extracts.
[c] Feedback-resistance was confirmed for concentrations up to 40 mM L-leucine.

### 3.2 Relevance of the repressor LtbR for L-leucine synthesis

The transcriptional regulator LtbR was shown to repress expression of *leuCD* and *leuB* in the presence of high L-leucine concentrations (Brune et al., 2007). Therefore, *ltbR* was deleted in the wild type to increase the expression of *leuCD* and *leuB* and the resulting strain Δ*ltbR* was transformed with pAN6-*leuA*_B018. The *leuCD* genes encode 3-isopropylmalate dehydratase (IPMD). In strain *C. glutamicum* Δ*ltbR*, an IPMD activity of 0.79 ± 0.06 µmol $min^{-1}$ $mg_{protein}^{-1}$ was measured, which corresponds to a 13-fold increase in comparison to the wild type (0.06 ± 0.01 µmol $min^{-1}$ $mg_{protein}^{-1}$). Strain *C. glutamicum* Δ*ltbR* showed slightly reduced growth (0.38 ± 0.01 $h^{-1}$) in comparison to the wild type but no L-leucine accumulation, whereas *C. glutamicum* Δ*ltbR* with pAN6-*leuA*_B018 produced 43.4 ± 1.1 mM L-leucine, which is approximately 10% more than wild-type *C. glutamicum* with pAN6-*leuA*_B018. As an additional control of this effect of the deletion of *ltbR*, also L-leucine accumulation using the other feedback-resistant IPMS_mod in *C. glutamicum* Δ*ltbR* was tested. With 32.6 ± 0.2 mM L-leucine produced in shake flask experiments, overexpression of pAN6-*leuA*_mod in *C. glutamicum* Δ*ltbR* showed a significantly higher L-leucine yield than when expressed in the wild-type background (26.1 ± 0.2 mM). Consequently, *C. glutamicum* Δ*ltbR* was used for further studies.

## 3.3 Construction of strains for increased *leuA* expression

### 3.3.1 Exchange of the native *leuA* promoter and its attenuator

In addition to the activity control of *leuA*-encoded IPMS, *leuA* expression is controlled by attenuation (Mentz et al., 2013; Pátek et al., 1994). In order to abolish premature termination of transcription, the native *leuA* promoter region and the attenuator sequence encoding the leader peptide were replaced by the strong promoter of the elongation factor TU ($P_{tuf}$) (Becker et al., 2005). Since several attempts of a direct replacement failed, *leuA* was first deleted together with its entire promoter region in *C. glutamicum* Δ*ltbR* to generate the leucine-auxotrophic strain *C. glutamicum* Δ*ltbR* Δ*leuA*. This strain was transformed with pK19*mobsacB*-$P_{tuf}$-*leuA*_B018 carrying the *leuA*_B018 gene under the control of $P_{tuf}$ and the native ribosome-binding site of the *tuf* gene. By selection for regain of growth on leucine-free minimal medium, the requested strain was obtained and the correct integration verified by sequencing. This strain, MV-Leu20, accumulated approximately 20 mM L-leucine within 24 h in CGXII-glucose (Figure 4). The IPMS enzyme activity of strain MV-Leu20 was 0.09 ± 0.01 µmol $min^{-1}$ $mg_{protein}^{-1}$, corresponding to a 50% increase compared to the activity of the wild type. Although the increase was lower than expected, replacement of the *leuA* leader region by $P_{tuf}$ had a significant positive influence on product formation.

### 3.3.2 Integration of a second *leuA* copy

Since the IPMS enzyme activity was still quite low in strain MV-Leu20, a second copy of $P_{tuf}$-*leuA*_B018 was integrated into the Δ*ltbR* locus of MV-Leu20. The resulting strain MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 had an IPMS activity of 0.18 ± 0.01 µmol $min^{-1}$ $mg_{protein}^{-1}$ and L-leucine accumulation was increased by approximately 25% to a titer of 26.4 ± 1.0 mM. Both strains MV-Leu20 and MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 were used for further improvements of L-leucine production.

## 3.4 Balancing precursor supply and metabolic flux towards L-leucine

### 3.4.1 Use of feedback-resistant acetohydroxyacid synthase

The specific IPMS activity in the strain MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 was almost twice as high as the glucose uptake rate of *C. glutamicum* of approximately 0.1 µmol $min^{-1}$ $mg_{protein}^{-1}$. This uptake rate is in accordance with the value given by Frunzke et al. (2008) for the wild-type strain. Therefore, it was intended to increase the precursor supply, which was already achieved in L-valine producers by increasing acetohydroxyacid synthase (AHAS) activity

(section 1.4). To this end, the *ilvN* gene encoding the regulatory subunit of AHAS was exchanged in MV-Leu20 and MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 by a variant encoding IlvN_fbr carrying amino acid exchanges G20D, I21D, and I22F (Elišáková et al., 2005). The resulting strain originating from MV-Leu20, MV-ValLeu33, produced approximately 33 mM L-leucine and 30 mM L-valine after 28 h cultivation in CGXII-glucose (Figure 4). The strain originating from MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 produced up to 55 mM L-leucine and just approximately 8 mM L-valine (Figure 4). The latter strain was designated MV-Leu55.

### 3.4.2 Analysis of limitations of L-leucine production in strain MV-Leu55

The strain comparison and the strong increase of L-leucine obtained showed that both, improved precursor supply ("push") and increased IPMS activity to redirect metabolic flux towards L-leucine instead L-valine ("pull"), offer potential for a further increase. However, attempts to integrate a third copy of the construct $P_{tuf}$-*leuA*_B018 into MV-Leu55 failed. Plasmid-based expression of *leuCD*, *leuB*, *ilvE* and *brnFE in* MV-Leu55 did not yield higher L-leucine titers, and only traces of 2-ketoisocaproate (KIC) were detected in culture supernatants (data not shown). This suggests that the activities of the proteins encoded by these genes are not limiting L-leucine production in the MV-Leu55 strain background. Interestingly, overexpression of *leuA*_B018 via plasmid pAN6-*leuA*_B018 in *C. glutamicum* Δ*ltbR ilvN*_fbr yielded only approximately 45 mM L-leucine, which is 10 mM less than using two copies of *leuA*_B018 under control of the $P_{tuf}$ promoter (data not shown). Besides this, also a deletion of *brnQ*, encoding the importer for all three BCAAs (Tauch et al., 1998), showed no effect in MV-Leu55 by preventing a possible re-uptake of L-leucine from the supernatant (data not shown). An undesired characteristic of MV-Leu55 was the formation of L-valine as a by-product. Therefore, *avtA* was deleted in MV-Leu55 since the encoded transaminase AvtA has the highest activity for L-valine formation (Marienhagen et al., 2005). However, no difference in product and by-product accumulation was found (data not shown).

### 3.4.3 Analysis of the growth behavior of MV-Leu55

Another characteristic of strain MV-Leu55 was that it exhibited two different growth phases: In the first 10 h a growth rate of about 0.18 $h^{-1}$ was observed, which decreased to approximately 0.09 $h^{-1}$ between 10 h and 32 h (Figure 4). This led to a prolonged cultivation time of up to 52 h to reach maximal L-leucine titers and consequently to a reduction in volumetric productivity. It was investigated if the reduced growth rate in the second phase was due to a negative effect of increased L-leucine concentrations on growth. Addition of 40 mM L-leucine to strain MV-Leu55 right from the beginning of the cultivation did not change

its growth behavior or productivity (data not shown), showing that other, yet unknown reasons are responsible for the bi-phasic growth behavior.

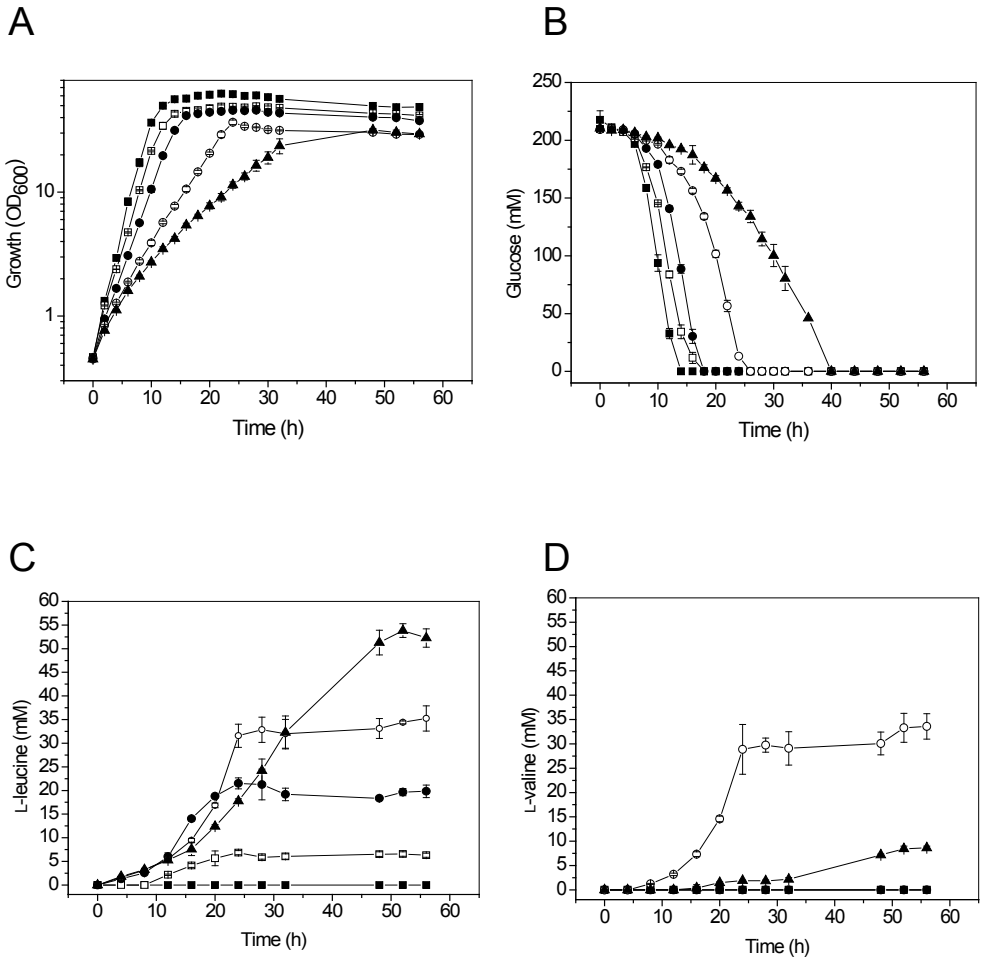



**Figure 4. Comparison of the *C. glutamicum* strains wild type (■), MV-Leu7 (□), MV-Leu20 (●), MV-ValLeu33 (○), and MV-Leu55 (▲) during cultivation in shake flasks with CGXII minimal medium and 4% (w/v) glucose.**

Growth (A), glucose consumption (B), L-leucine formation (C), and L-valine formation (D) are shown. The data represent mean values and standard deviations obtained from three independent cultivations.

### 3.5 Genome wide gene expression analysis

In order to confirm the increased expression of genes responsible for L-leucine-biosynthesis and to search for additional targets for strain improvement, comparative transcriptome analyses were performed with DNA microarrays. Strain MV-Leu55 was compared both with *C. glutamicum* wild type and with strain MV-Leu20. The experiments revealed 15 strongly differentially expressed genes (ratio of medians < 0.25 or > 4.0, *p*-value < 0.05) in at least one of the two comparisons (Table 7). As expected, *leuA*, *leuB*, and *leuCD* showed significant upregulation (factor 4-9) when comparing strain MV-Leu55 with the wild type, whereas the comparison of MV-Leu55 with MV-Leu20 showed most notably an upregulation of *leuA*. In addition, the *brnFE* genes, encoding an exporter of branched-chain amino acids (Kennerknecht et al., 2002), was upregulated in strain MV-Leu55, apparently as a consequence of increased cytosolic L-leucine concentrations to enhance its export by BrnFE. Most prominent was the expression change of the gene cg3022, which was more than 14-fold and 8-fold upregulated in MV-Leu55 as compared to *C. glutamicum* wild type or MV-Leu20, respectively. The amino acid sequence of Cg3022 shares significant similarity to the C-terminal regulatory domain of IPMS (http://toolkit.tuebingen.mpg.de/hhpred). When cg3022 was deleted in MV-Leu55, there was no change of the L-leucine titer, but the L-valine titer increased from about 8 mM to 19.3 ± 0.7 mM. Thus, the protein encoded by cg3022 appears to be involved somehow in the synthesis of BCAAs, but is not necessary to obtain high L-leucine formation. No obvious additional targets for the improvement of L-leucine biosynthesis could be identified. Therefore, other strategies to improve productivity and decrease formation of by-products of strain MV-Leu55 were used.

**Table 7. Differentially expressed genes in transcriptome comparisons of MV-Leu55/ *C. glutamicum* wild type and MV-Leu55/MV-Leu20 using DNA microarray experiments[a,b].**

| Gene | Annotation | MV-Leu55 / wild type | | MV-Leu55 / MV-Leu20 | |
|---|---|---|---|---|---|
| | | relative mRNA level | *p*-value | relative mRNA level | *p*-value |
| **cg0286** | putative membrane protein, conserved | 0.14 | 0.015 | 0.16 | 0.052 |
| **cg3427** | *parA1*, ATPase involved in chromosome partitioning | 0.14 | 0.020 | 0.92 | 0.136 |
| **cg0791** | *pyc*, pyruvate carboxylase | 0.15 | 0.005 | 0.54 | 0.125 |

| Gene | Annotation | MV-Leu55 / wild type | | MV-Leu55 / MV-Leu20 | |
|---|---|---|---|---|---|
| | | relative mRNA level | *p*-value | relative mRNA level | *p*-value |
| **cg1364** | *atpF*, $F_1F_0$-ATP synthase b-subunit of $F_0$ part | 0.15 | 0.019 | 0.98 | 0.463 |
| **cg1366** | *atpA*, $F_1F_0$-ATP synthase α-subunit of $F_0$ part | 0.15 | 0.040 | 0.94 | 0.372 |
| **cg2184** | ATPase component of peptide ABC-type transport system, contains duplicated ATPase domains | 0.41 | 0.002 | 0.19 | 0.010 |
| **cg1419** | putative $Na^+$-dependent transporter, bile acid:$Na^+$ symporter BASS family | 4.24 | 0.019 | 0.68 | 0.008 |
| **cg2636** | *catA1*, catechol 1,2-dioxygenase | 4.33 | 0.013 | 4.84 | 0.012 |
| **cg0303** | *leuA*, 2-isopropylmalate synthase | 4.86 | 0.002 | 3.10 | 0.098 |
| **cg1487** | *leuC*, 3-isopropylmalate dehydratase large subunit | 6.83 | 0.004 | 1.92 | 0.174 |
| **cg0315** | *brnE*[c], branched-chain amino acid exporter, small subunit | 7.05 | 0.034 | 1.42 | 0.296 |
| **cg1488** | *leuD*, 3-isopropylmalate dehydratase small subunit | 7.64 | 0.022 | 1.00 | 0.470 |
| **cg1658** | putative permease of the major facilitator superfamily | 8.77 | 0.046 | 0.84 | 0.025 |
| **cg1453** | *leuB*, 3-isopropylmalate dehydrogenase | 9.45 | 0.005 | 1.00 | 0.320 |
| **cg3022** | putative acetyl-CoA acetyltransferase, conserved | 14.00 | 0.002 | 8.61 | 0.005 |

[a] Genes were chosen which fulfilled the criteria relative mRNA level > 4 or < 0.25 in at least one of the two comparisons.
[b] Data of each comparison represent mean values of three independent DNA microarray experiments starting from independent cultures grown in CGXII minimal medium + 4% (w/v) glucose.
[c] Relative mRNA levels of cg0314 (*brnF,* annotated as branched chain amino acid exporter, large subunit) were 2.50 (*p*-value 0.006) and 1.60 (*p*-value 0.048) for comparison of MV-Leu55/wild type and MV-Leu55/MV-Leu20, respectively.

## 3.6 Optimizing productivity of MV-Leu55

### 3.6.1 Increase of glucose uptake

It was demonstrated that deletion of the transcriptional regulator IolR derepresses genes of inositol metabolism in *C. glutamicum* (Klaffl et al., 2013), including *iolT1* encoding the PTS-independent inositol transporter IolT1 which also catalyzes glucose uptake (Ikeda et al., 2011; Lindner et al. 2011). Assuming that an increased glucose uptake capacity could improve growth and production properties of MV-Leu55, *iolR* was deleted. The resulting strain MV-Leu55 Δ*iolR* exhibited an increased and constant growth rate of 0.2 $h^{-1}$. The glucose uptake rate was increased by 43% from 53 ± 3 $nmol^{-1}\,min^{-1}\,mg_{CDW}^{-1}$ in strain MV-Leu55 up to 76 ± 1 $nmol^{-1}\,min^{-1}\,mg_{CDW}^{-1}$ in strain MV-Leu55 Δ*iolR*. Despite the fact that the L-leucine titer was decreased to approximately 40 mM and at the same time the L-valine titer was increased to approximately 20 mM (Figure 5), volumetric productivity was strongly increased by about 40% from 1.04 ± 0.03 mmol $l^{-1}\,h^{-1}$ to 1.38 ± 0.02 mmol $l^{-1}\,h^{-1}$ (Figure 6). Besides increased productivity, MV-Leu55 Δ*iolR* showed other benefits: growth and product accumulation turned out to be exhibiting exceptional reproducibility, probably due to a well-balanced precursor supply based on increased sugar uptake. However, compared to its precursor strain MV-Leu55, it showed increased by-product formation and a lower L-leucine titer. The added total concentration of L-valine and L-leucine remained the same as in MV-Leu55.

### 3.6.2 Integration of a third *leuA* copy

It was assumed that the flux towards KIV is increased in MV-Leu55 Δ*iolR* and IPMS activity seems to be insufficient to convert this precursor completely to L-leucine. Therefore, another attempt was made to integrate a third copy of $P_{tuf}$-*leuA*_B018 into the intergenic region between cg1121 and cg1122 to reduce L-valine formation in favor of increased L-leucine titer and yield. The intergenic region between these divergently organized genes in the genome of *C. glutamicum* was successfully used in the past for integration of genes or operons (Blombach et al., 2009). In the MV-Leu55 Δ*iolR* background, integration of a third copy of P*tuf*-*leuA*_B018 was achieved, perhaps due to the increased glucose uptake capacity of strain MV-Leu55 Δ*iolR*. As expected, the resulting strain MV-LeuF1 showed an increased L-leucine titer of approximately 45 mM, while formation of L-valine was reduced to 15 mM (Figure 5). Compared to strains with two copies of $P_{tuf}$-*leuA*_B018, the IPMS activity of MV-LeuF1 was increased by about 10% to 0.20 ± 0.01 µmol $min^{-1}\,mg_{protein}^{-1}$ (Table 8).

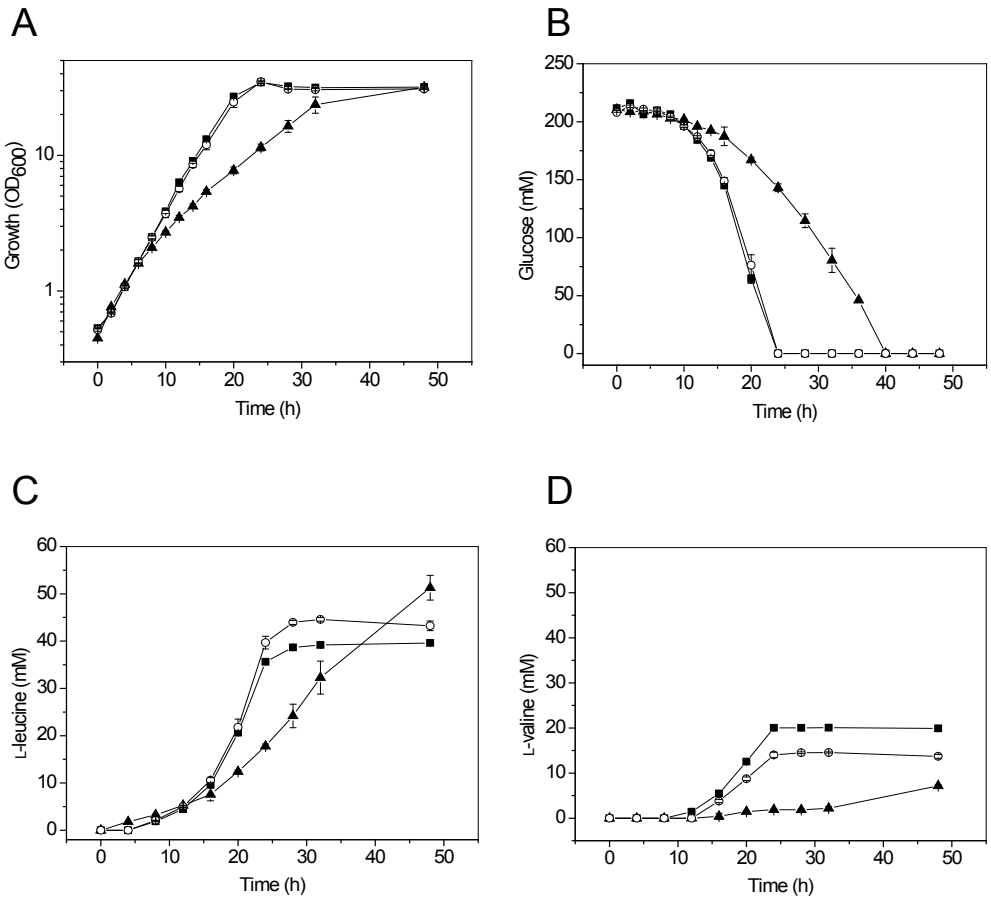



**Figure 5. Comparison of the *C. glutamicum* strains MV-Leu55 (▲), MV-Leu55 Δ*iolR* (■), and MV-LeuF1 (○) during cultivation in shake flasks with CGXII minimal medium and 4% (w/v) glucose.**

Growth (A), glucose consumption (B), L-leucine formation (C), and L-valine formation (D) are shown. The data represent mean values and standard deviations obtained from three independent cultivations.

**Table 8. Specific 2-isopropylmalate synthase activities of plasmid-free L-leucine producers with different copy numbers of the module $P_{tuf}$-*leuA*_B018 obtained in shake flask cultivations in CGXII minimal medium with 4% (w/v) glucose[a].**

| Strain | Number of $P_{tuf}$-*leuA*_B018 copies | Specific IPMS activity (µmol $min^{-1}$ $mg_{protein}^{-1}$) |
|---|---|---|
| MV-Leu20 | 1 | 0.09 ± 0.01 |
| MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 | 2 | 0.18 ± 0.01 |
| MV-LeuF1 | 3 | 0.20 ± 0.01 |

[a] All data represent mean values with standard deviations obtained from at least three independent measurements with different crude extracts.

### 3.7 Reduction of citrate synthase activity

As strain MV-LeuF1 was not in shortage of the IPMS substrate KIV due to the observed L-valine accumulation, it was decided to increase the supply of the second IPMS substrate acetyl-CoA. To this end, it was made use of the previous observation that the reduction of citrate synthase activity in *C. glutamicum* is beneficial for L-lysine production and also leads to an increase in the intracellular concentration of acetyl-CoA (van Ooyen et al., 2012). Consequently, the two promoters in front of the *gltA* gene encoding citrate synthase (van Ooyen et al., 2011), were replaced by the weaker promoter $P_{dapA\text{-}L1}$ (Vašcicová et al., 1999) in MV-LeuF1, which reduces the citrate synthase activity to 16% of the wild-type activity (van Ooyen et al., 2012). The resulting strain MV-LeuF2 was comparable to MV-LeuF1 with respect to growth, but L-leucine formation was increased to 52.0 ± 1.9 mM and L-valine formation was strongly decreased to 4.6 ± 0.1 mM. These results confirmed the successful redirecting of the KIV flux from L-valine towards L-leucine due to increased acetyl-CoA supply. The L-leucine volumetric productivity of MV-LeuF2 was increased to 1.73 ± 0.07 mmol $l^{-1}$ $h^{-1}$ (Figure 6).

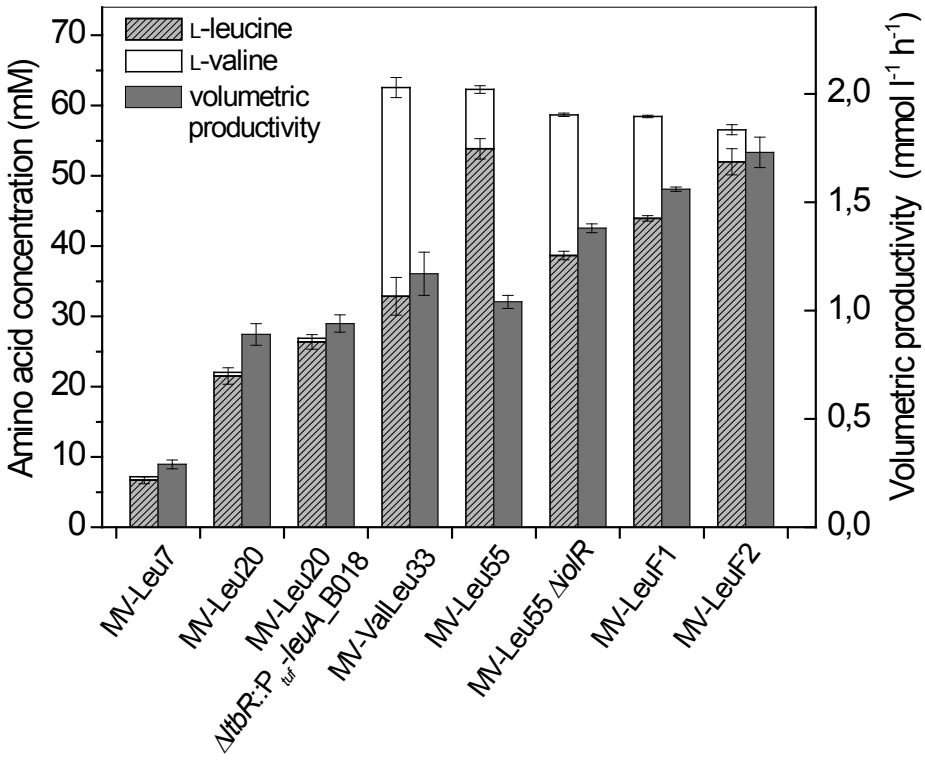



**Figure 6. Maximal values of L-leucine formation, L-valine formation, and volumetric productivity obtained for the indicated L-leucine producing strains in shake flask experiments**.

L-Leucine titers were reached after 32 h at the latest, except for strain MV-Leu55 which reached the maximal concentration after 52 h. The data represent mean values and standard deviations obtained from three independent cultivations.

### 3.8 Relationship between growth rate and L-leucine titer

Since the precursors for building blocks are used for biomass and product formation as well, the growth rates of the different L-leucine production strains were compared (Table 9). Indeed, with the exception B018, there is a clear correlation of increasing L-leucine titer with decreasing growth rate. Strain B018 was obtained by undirected mutagenesis and is likely to carry additional mutations which are disadvantageous for growth. The observed correlation of increasing L-leucine formation with decreasing growth is a strong indication for a limited supply of precursors. This agrees with the positive effect on the growth rate when the sugar uptake is increased by *iolR* deletion (Ikeda et al., 2011).

**Table 9. Growth rates and L-leucine titers obtained with the indicated strains in shake flask cultivations in CGXII minimal medium with 4% (w/v) glucose[a].**

| Strain | Growth rate ($h^{-1}$) | L-leucine titer (mM) |
|---|---|---|
| *C. glutamicum* wild type | 0.44 ± 0.01 | 0 |
| B018 | 0.23 ± 0.01 | 7.8 ± 2.0 |
| Δ*ltbR* | 0.38 ± 0.01 | 0 |
| MV-Leu7 | 0.38 ± 0.01 | 6.9 ± 0.5 |
| MV-Leu20 | 0.31 ± 0.01 | 21.5 ± 1.2 |
| MV-Leu20 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 | 0.25 ± 0.01 | 26.4 ± 1.0 |
| MV-ValLeu33 | 0.21 ± 0.01 | 32.9 ± 2.7 |
| MV-Leu55 | 0.09 - 0.18[b] | 53.9 ± 1.5 |
| MV-Leu55 Δ*iolR* | 0.20 ± 0.01 | 38.7 ± 0.6 |
| MV-LeuF1 | 0.20 ± 0.01 | 44.0 ± 0.4 |
| MV-LeuF2 | 0.19 ± 0.01 | 52.0 ± 1.9 |

[a] The data represent mean values and standard deviations obtained from three independent cultivations.
[b] MV-Leu55 showed an initial growth rate of approximately 0.18 $h^{-1}$ in the first 10 h, which decreased to approximately 0.09 $h^{-1}$ between 10 h and 32 h.

All relevant metabolic engineering steps performed in the different plasmid-free L-leucine producers of *C. glutamicum* up to MV-LeuF2 are summarized and depicted in Figure 7.

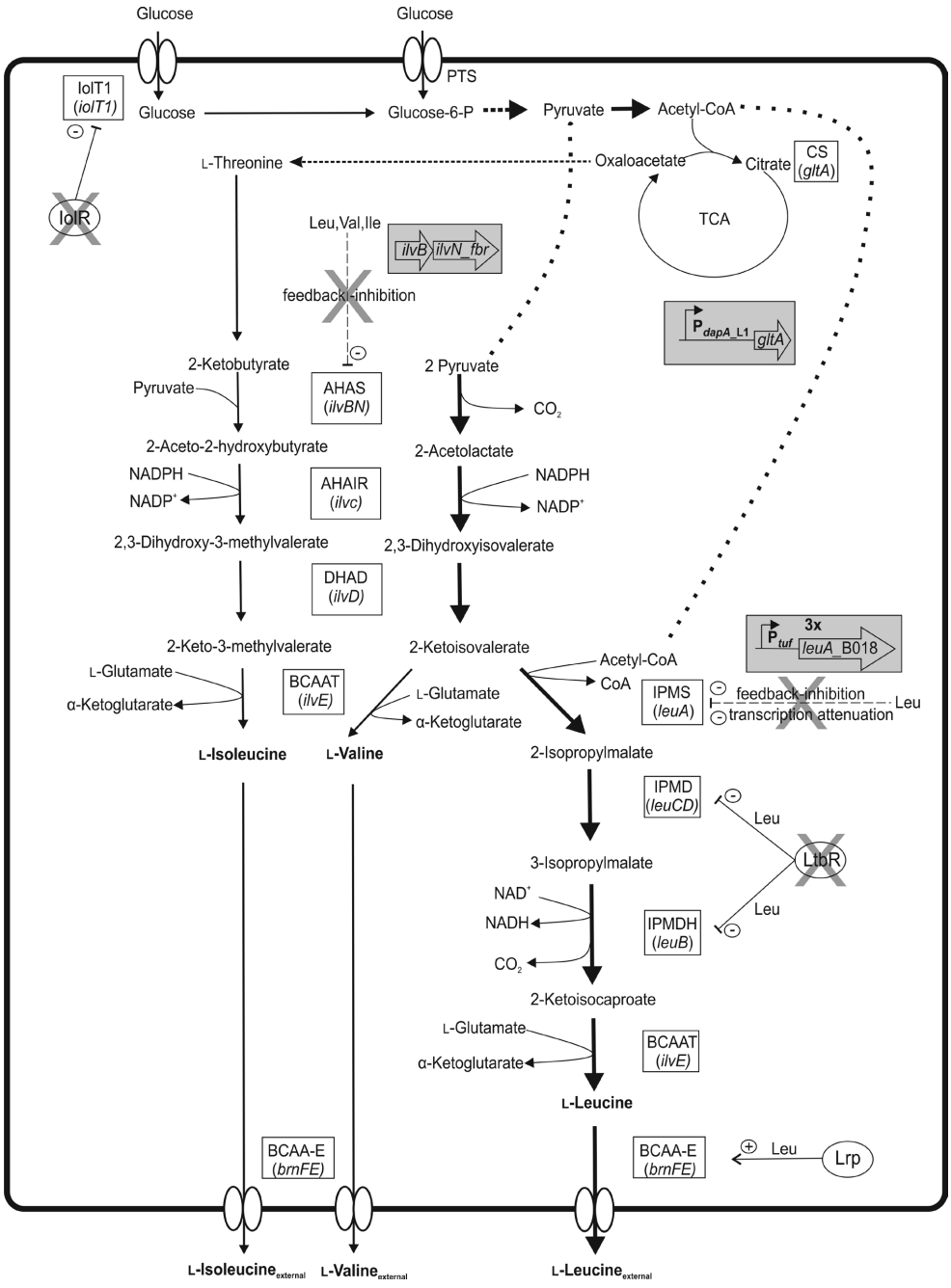



**Figure 7. Schematic representation of the L-leucine biosynthesis pathway of *C. glutamicum* and of the metabolic engineering steps performed in this work.**

Enzymes and their corresponding genes relevant for this work are shown in boxes. Regulatory proteins are shown in cycles. Lines with "+" indicate activation of gene expression; "-" indicates repression of gene expression (solid lines) or indicates feedback-

inhibition/transcription attenuation (dashed lines). “Leu”, “Val”, and Ile” indicate the presence of L-leucine, L-valine, and L-isoleucine, respectively. Deletion of genes and the respective proteins as well as abolishment of feedback-inhibition and transcription attenuation are indicated by “X”. Metabolic engineering steps, i.e. promoter exchanges and integration of genes encoding feedback-resistant enzyme variants, are highlighted in grey. Thick arrows indicate increased metabolic fluxes. Abbreviations: AHAS, acetohydroxyacid synthase; AHAIR, acetohydroxyacid isomeroreductase; BCAA-E, branched-chain amino acid exporter (BrnFE); BCAAT, branched-chain amino acid transaminase IlvE; CS, citrate synthase; DHAD, dihydroxyacid dehydratase; fbr, feedback-resistant; Glucose-6-P, glucose-6-phosphate; IPMD, 3-isopropylmalate dehydratase; IPMDH, 3-isopropylmalate dehydrogenase; IPMS, 2-isopropylmalate synthase; Lrp, leucine-responsive protein; LtbR, leucine and tryptophane biosynthesis regulator; P, promoter; PTS, phosphotransferase system; TCA, tricarboxylic acid cycle.

## 3.9 Batch and fed-batch cultivations

### 3.9.1 Test fermentations

Strains MV-Leu55 and MV-LeuF1 were characterized in preliminary batch fermentations (pH 7.2). MV-LeuF1 showed a production pattern similar to that obtained in shake flask experiments (Table 10). In contrast, MV-Leu55 exhibited slow growth ($\mu$ = 0.12 ± 0.2 $h^{-1}$) and significantly increased L-valine formation as a by-product (12.8 ± 0.6 mM) at the cost of reduced L-leucine accumulation (34.4 ± 0.9 mM). Notably, the slow growth of MV-Leu55 was constant (data not shown), without further decrease during proceeding cultivation time as obtained in shake flask cultivations (Figure 4). Nevertheless, the main advantage of MV-Leu55 - reaching the highest titer of all strains in shake flask cultivations - was absent in bioreactor fermentations. Therefore, it was decided to use MV-LeuF1 and MV-LeuF2 for further improvement of fermentation conditions.

### 3.9.2 Optimizing fermentation conditions

Strains MV-LeuF1 and MV-LeuF2 were characterized in fed-batch cultivations in order to determine the influence of different process parameters on productivity and to analyze the behavior of the strains under such controlled conditions. In addition to L-valine, also L-alanine and L-lysine accumulated as by-products in initial fed-batch-experiments at pH 7.2 to concentrations exceeding 24 mM and 10 mM, respectively, especially in the fed-batch phase. To determine the optimal pH for L-leucine production, cultivations were performed at pH 6.5, 7.2, and 8.0. At pH 8.0, cells stopped growing when they reached an $OD_{600}$ of 4-5 and therefore this pH was not studied any further (data not shown). Table 10 shows product and by-product formation of MV-LeuF1 at pH values of 6.5 and 7.2. Cells showed similar growth rates at both pH values, but product and by-product formation differed. In comparison to

pH 7.2, L-lysine secretion was increased at pH 6.5 by approximately 45% to 14 mM, but the L-valine and L-alanine titers were reduced by 65% and 30% to approximately 6 mM and 17 mM, respectively. Moreover, L-leucine formation was increased at pH 6.5 in the batch phase by approximately 20% compared to pH 7.2 (from 33 mM to 40 mM). Based on these results, the subsequent fermentations were performed at pH 6.5. Furthermore, the feed solution, which originally contained only 50% (w/v) glucose, was supplemented with 4% (w/v) ammonium sulfate, as increasing concentrations of KIC were detected in the culture supernatant at later time points of the fed-batch process, indicating nitrogen limitation.

**Table 10. Influence of pH values 6.5 and 7.2 on product and by-product formation in fed-batch fermentations of strain MV-LeuF1[a,b].**

| | **Batch phase** | |
|---|---|---|
| | pH 6.5 | pH 7.2 |
| Cultivation time (h)[c] | 25.0 | 25.0 |
| L-Leucine (mM) | 39.5 ± 0.1 | 32.8 ± 0.1 |
| By-products | | |
| L-Valine (mM) | 5.1 ± 0.1 | 10.8 ± 0.3 |
| L-Alanine (mM) | 2.7 ± 0.1 | 3.8 ± 0.6 |
| L-Lysine (mM) | 4.7 ± 0.0 | 3.9 ± 0.1 |
| | **Batch phase + Feed phase** | |
| | pH 6.5 | pH 7.2 |
| Cultivation time (h) | 54.0 | 54.0 |
| L-leucine (mM) | 127.4 ± 2.1[d] | 128.5 ± 11.5[d] |
| By-products | | |
| L-Valine (mM) | 5.9 ± 0.4 | 16.8 ± 1.7 |
| L-Alanine (mM) | 16.8 ± 1.0 | 24.4 ± 0.1 |
| L-Lysine (mM) | 13.9 ± 1.0 | 9.5 ± 0.1 |

[a] Fed-batch fermentations were run with 600 ml (modified) CGXII minimal medium with 4% (w/v) glucose. Feed phase was performed with feed solution containing 50% (w/v) glucose. The overall process was run for 54 h.
[b] The values given are the average data with standard deviations from two independent fed-batch fermentations.
[c] The growth rates in batch phase for pH 6.5 and pH 7.2 were 0.15 ± 0.01 $h^{-1}$ and 0.14 ± 0.01 $h^{-1}$, respectively.
[d] A few mmol of the L-leucine precursor KIC were also detected in the supernatants, indicating nitrogen source limitation.

### 3.9.3 Characteristics of fed-batch cultivations of MV-LeuF1 and MV-LeuF2

Using the optimized conditions described above, three independent fed-batch cultivations were performed with each of the strains MV-LeuF1 and MV-LeuF2. Representative experiments are shown in Figure 8 and Figure 9, respectively; the additional cultivations are presented in Figure 10. The average process data of all three fed-batch cultivations of MV-LeuF1 and MV-LeuF2 are listed in Table 11.

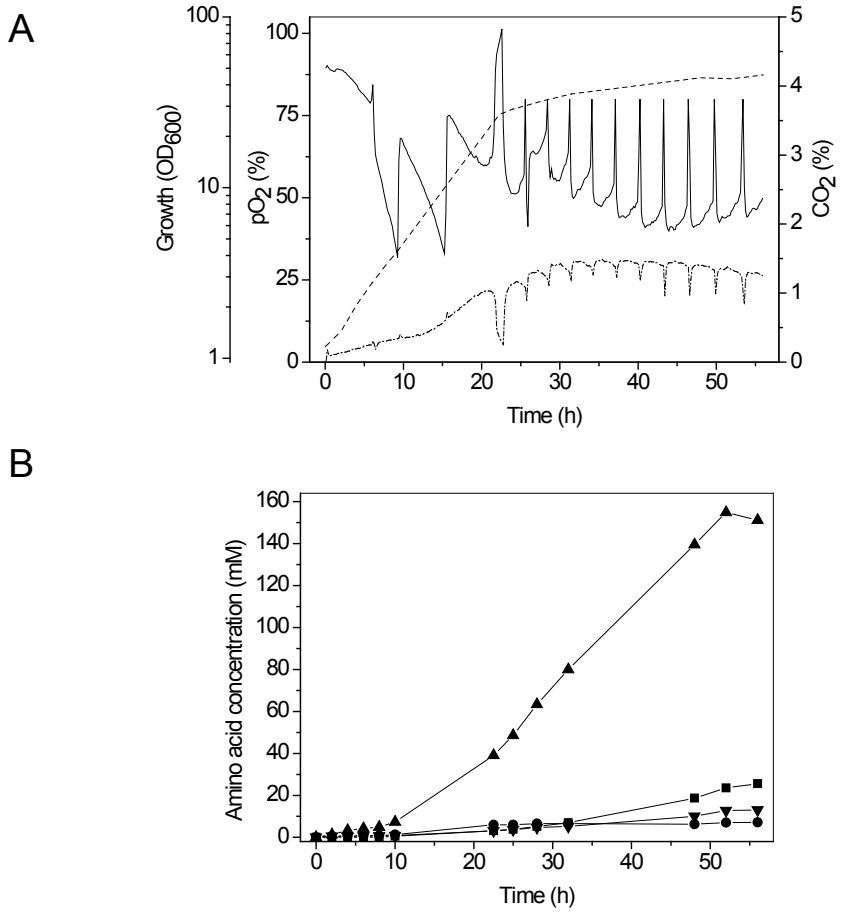



**Figure 8. Representative fed-batch fermentation of MV-LeuF1.**

Panel A shows growth (dashed line), oxygen partial pressure ($pO_2$) representing the dissolved oxygen saturation (solid line), and absolute $CO_2$ concentration (dashed/dotted line). Panel B shows the formation of L-leucine (▲), L-valine (●), L-alanine (■), and L-lysine (▼). The Multifors bioreactor was filled with 500 ml modified CGXII medium containing 4% (w/v) glucose for the batch phase. In the feed phase, an aqueous feeding solution containing 50% glucose (w/v) and 4% (w/v) ammonium sulfate was used. The pH was kept constant at value 6.5 by addition of 3 M HCl or 3 M NaOH. Decreasing $pO_2$ and rising $CO_2$

signals were due to the respiratory activity of the growing cells. A sudden rise of $pO_2$ in the first hours of the cultivation (here after approximately 10 h and 16 h) is due to an increase of the stirrer speed in order to maintain $pO_2$ > 30%. The batch phase ended after 22.5 h due to depletion of glucose as indicated by an increase of pO2 to > 80%, followed by the start of the feed phase. The automatic feeding program created a “sawtooth” pattern in the feed phase: a sudden rise of $pO_2$ to a value of > 80% indicated the depletion of glucose and triggered the addition of feed solution. This in turn led to a drop of $pO_2$ due to the restarting metabolic activity of the cells. The cultivation was stopped after 56 h.

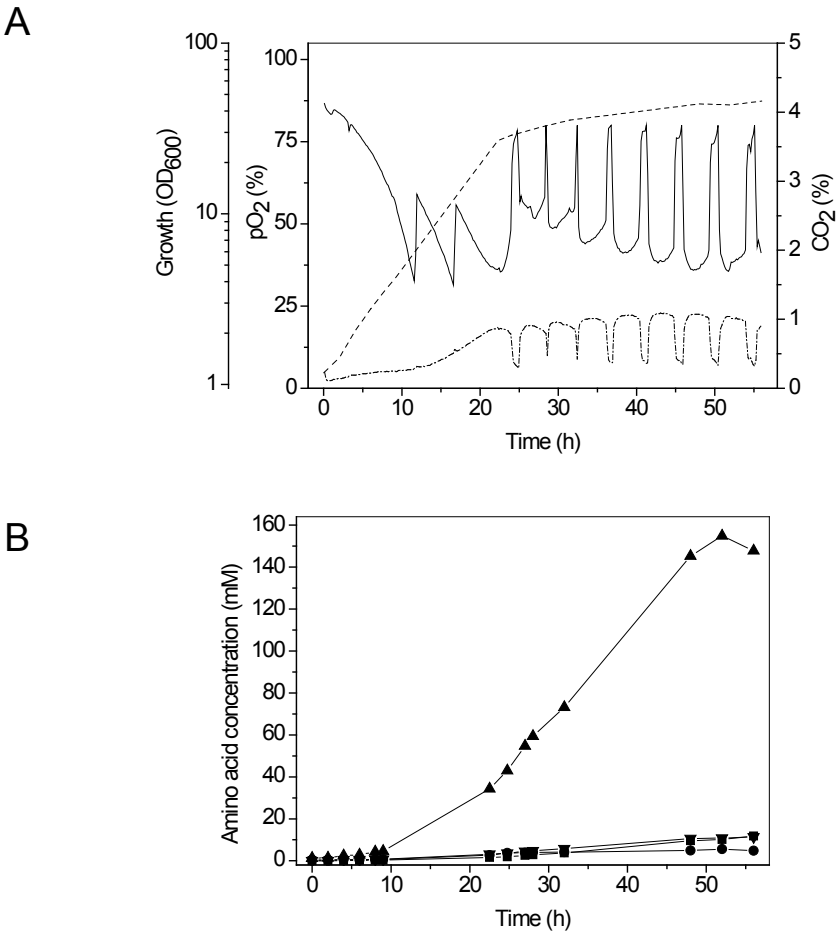



**Figure 9. Representative fed-batch fermentation of MV-LeuF2.**

Panel A shows growth (dashed line), oxygen partial pressure ($pO_2$) representing the dissolved oxygen saturation (solid line), and absolute $CO_2$ concentration (dashed/dotted line). Panel B shows the formation of L-leucine (▲), L-valine (●), L-alanine (■), and L-lysine (▼). For details see legend of Figure 8. The batch phase ended/feed phase started after 24.5 h. The cultivation was stopped after 56 h.

Strain MV-LeuF1 produced 151.2 ± 5.1 mM (approximately 20 g $l^{-1}$) L-leucine within 52 h with a molar product yield (mol L-leucine/mol glucose) of 0.253 ± 0.009 in the feed phase and 0.233 ± 0.005 in the overall process. The volumetric productivity was 3.9 ± 0.1 mmol $l^{-1}$ $h^{-1}$ in the feed stage and 2.9 ± 0.1 mmol $l^{-1}$ $h^{-1}$ in the overall process. The by-product concentrations were relatively small: 7.5 ± 0.4 mM L-valine, 11.7 ± 1.2 mM L-lysine, and 20.2 ± 4.3 mM L-alanine in the overall process. Strain MV-LeuF2 reached the same final L-leucine titer of 151.9 ± 12.7 mM, but the concentrations of the by-products L-valine (5.8 ± 1.2 mM) and L-alanine (11.0 ± 1.0 mM) were lower, resulting in an increased molar product yield of 0.298 ± 0.013 mol per mol of glucose in the feed phase and 0.263 ± 0.012 mol per mol of glucose in the overall process. The volumetric productivity of MV-LeuF2 in the feed phase (4.3 ± 0.4 mmol $l^{-1}$ $h^{-1}$) and in the overall process (3.1 ± 0.3 mmol $l^{-1}$ $h^{-1}$) was also higher than that of strain MV-LeuF1.

When the process time in the fed-batch cultivations exceeded approximately 56 h, the L-leucine concentrations increased only slightly with both strains (Figure 8 and Figure 9). This effect could be explained by the beginning precipitation of the product L-leucine, which is very hydrophobic. Its solubility in water was reported to be 24.3 g $l^{-1}$ (Dawson, 1986). After 72 h of cultivation, thick foam and white powder on top of the cultures was observed, which was found to be almost pure L-leucine without other contaminating amino acids. The highest concentration of soluble L-leucine at this point was 181 mM (23.7 g $l^{-1}$), which corresponds quite exactly to the solubility value given above.

**Table 11. Results of fed-batch cultivations[a] of strains MV-LeuF1 and MV-LeuF2 including maximum L-leucine and by-product concentrations as well as specific molar yields and volumetric productivities of batch phase, feed phase, and the overall process[b].**

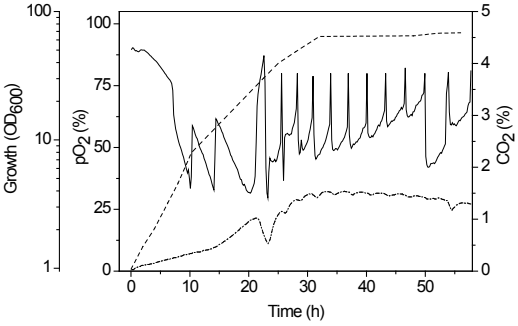
| | Batch phase[c] | | Feed phase | | Overall process (Batch + Feed phase) | |
|---|---|---|---|---|---|---|
| | MV-LeuF1 | MV-LeuF2 | MV-LeuF1 | MV-LeuF2 | MV-LeuF1 | MV-LeuF2 |
| Cultivation time (h) | 23.75 ± 1.25 | 24.00 ± 1.50 | 28.25 ± 1.25 | 25.00 ± 2.25 | 52.00 ± 0.50 | 50.00 ± 2.00 |
| L-Leucine (mM) | 39.0 ± 1.1 | 42.9 ± 2.1 | 112.2 ± 6.0 | 109.0 ± 11.5 | 151.2 ± 5.1 | 151.9 ± 12.7 |
| By-products: | | | | | | |
| L-Valine (mM) | 5.1 ± 0.7 | 4.2 ± 0.4 | 2.4 ± 0.3 | 1.6 ± 1.6 | 7.5 ± 0.4 | 5.8 ± 1.2 |
| L-Alanine (mM) | 2.9 ± 0.7 | 2.1 ± 0.2 | 17.3 ± 4.7 | 8.9 ± 1.2 | 20.2 ± 4.3 | 11.0 ± 1.0 |
| L-Lysine (mM) | 3.3 ± 0.3 | 4.1 ± 0.6 | 8.4 ± 1.6 | 7.6 ± 1.8 | 11.7 ± 1.2 | 11.7 ± 1.3 |
| Molar product yield (mol L-leucine/ mol glucose) | 0.176 ± 0.005 | 0.194 ± 0.010 | 0.253 ± 0.009 | 0.298 ± 0.013 | 0.233 ± 0.005 | 0.263 ± 0.012 |
| Volumetric productivity (mmol L-leucine/ (l x h)) | 1.7 ± 0.1 | 1.8 ± 0.2 | 3.9 ± 0.1 | 4.3 ± 0.4 | 2.9 ± 0.1 | 3.1 ± 0.3 |

[a] Fed-batch fermentations were run at a constant pH of 6.5 starting with 500 ml (modified) CGXII minimal medium + 4% (w/v) glucose. Feed phase was performed with feed solution containing 50% (w/v) glucose and 4% (w/v) ammonium sulfate. Overall process was run for 48-52 h.

[b] Mean values and standard deviations from three independent fed-batch cultivations of each strain are shown.

[c] The growth rates in batch phase for MV-LeuF1 and MV-LeuF2 were $0.19 \pm 0.02\ h^{-1}$ and $0.15 \pm 0.02\ h^{-1}$, respectively.

IA

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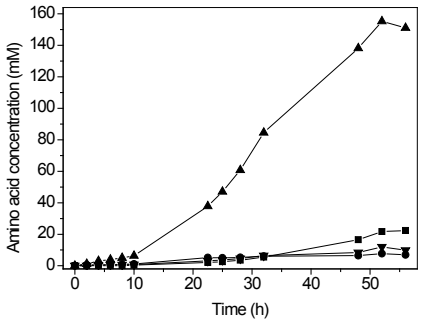


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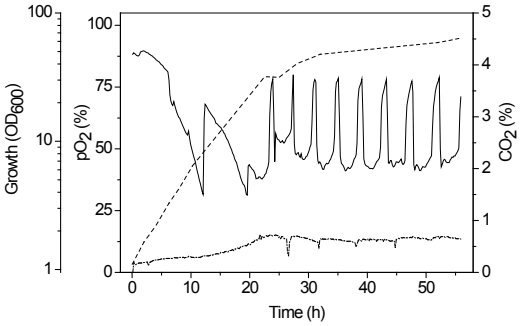


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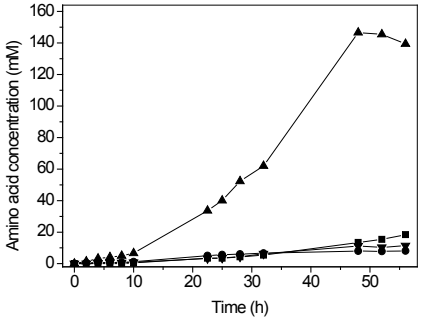


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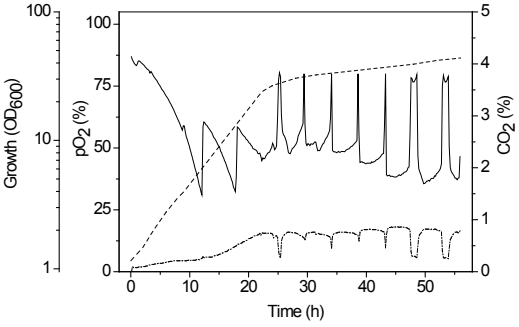


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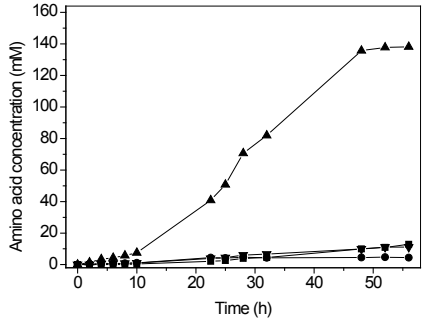

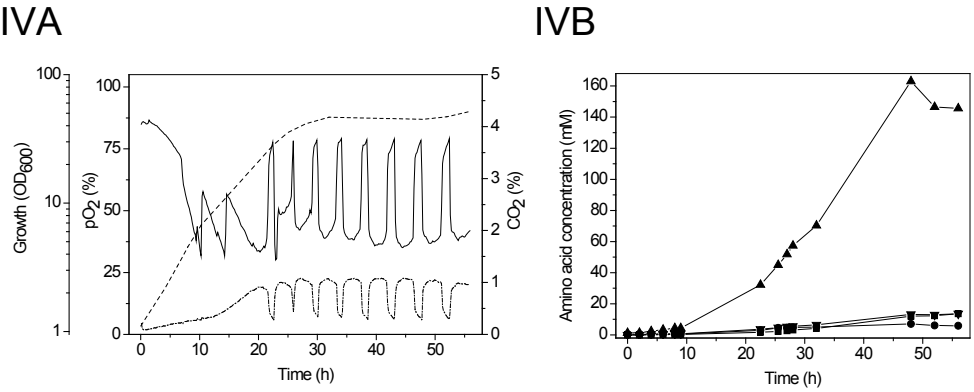


**Figure 10. Fed-batch fermentations of MV-LeuF1 (I+II) and MV-LeuF2 (III+IV).**

Panel A shows growth (dashed line), oxygen partial pressure ($pO_2$) representing the dissolved oxygen saturation (solid line), and absolute $CO_2$ concentration (dashed/dotted line). Panel B shows the formation of L-leucine (▲), L-valine (●), L-alanine (■), and L-lysine (▼). For details see legend of Figure 8 and Figure 9.

## 3.10 Production of 2-ketoisocaproate

### 3.10.1 Consequences of *ilvE* deletion at elevated L-leucine production

The next aim was to test if it was possible to turn L-leucine producers into producers of 2-ketoisocaproate (KIC). IlvE is the main transaminase involved in the conversion of KIC, 2-ketoisovalerate (KIV), and 2-keto-3-methylvalerate (KMV) into L-leucine, L-valine and L-isoleucine, respectively (Radmacher et al., 2002). The deletion of *ilvE* in *C. glutamicum* leads to auxotrophies for L-leucine and L-isoleucine, but not L-valine because this amino acid can be formed by transamination of KIV via the *avtA*-encoded enzyme (Marienhagen et al., 2005). It was expected that the deletion of the gene *ilvE* in an L-leucine producer would lead to accumulation of this precursor. The wild-type strain, the *C. glutamicum* Δ*ilvE* strain (supplemented with 1 mM L-leucine and 1 mM L-isoleucine) and the L-leucine producing strain *C. glutamicum* Δ*ltbR* with pAN6-*leuA*_B018 (induced with 0.1 mM IPTG) were tested in shake flask experiments with CGXII-glucose medium for KIC production. None of the strains were able to accumulate KIC in detectable concentrations in the supernatant. In a next step, *C. glutamicum* Δ*ilvE* was transformed with the plasmid pAN6-*leuA*_B018 and tested in CGXII-glucose supplemented with 1 mM L-leucine, 1 mM L-isoleucine, and 0.1 mM IPTG. This strain accumulated 34.0 ± 1.7 mM KIC in the supernatant, demonstrating that it is easily possible to convert a *C. glutamicum* L-leucine producer into a KIC producer.

### 3.10.2 Reduction of transamination activity in L-leucine producers

Analogously to the constructed L-leucine production strains, it was intended to design strains devoid of plasmids, heterologous genes, and auxotrophies for KIC production. Therefore, the aim was to downregulate the activity of IlvE instead of deleting *ilvE*. Low residual activity of IlvE should avoid auxotrophies for L-leucine and L-isoleucine, but still allow the accumulation of KIC. To reach this goal, the ATG start codon of *ilvE* should be exchanged against GTG start codon to decrease the translation initiation rate of the *ilvE* transcript, thereby reducing IlvE activity (Figure 11).

The ATG start codon of *ilvE* was exchanged against GTG start codon in MV-LeuF1, one of the most efficient L-leucine production strains, to yield strain MV-KICF1. Strain MV-KICF1 was cultivated in CGXII-glucose medium with or without 1 mM L-isoleucine and 1 mM L-leucine to monitor KIC accumulation in the supernatant. Remarkably, only the addition of L-isoleucine, but not L-leucine, was necessary to provide growth of MV-KICF1 (data not shown). Supplementation of L-leucine in addition to L-isoleucine for cultivation of MV-KICF1 did not alter the growth rate significantly in minimal medium so that addition of L-leucine was omitted in the cultivations. The direct ancestor strain of MV-KICF1 (MV-LeuF1) and strain SH-KIC20 (Sabine Haas, FZ Jülich) served as references (Figure 11). SH-KIC20 was constructed also by exchanging the ATG start codon of *ilvE* against GTG start codon in the L-leucine producer MV-Leu20. The results obtained for MV-KICF1 and SH-Leu20 are summarized in Table 12. Both SH-KIC20 and MV-KICF1 grew slowly without supplementation of L-isoleucine and confirmed that the start codon exchange provides residual IlvE activity to enable growth. The poor growth of these strains unfortunately led to very low volumetric KIC productivities of approximately 0.35 mmol $l^{-1}$ $h^{-1}$. Supplementation of L-isoleucine was additionally tested for MV-KICF1 and enabled this strain ($\mu$ = 0.21 ± 0.01 $h^{-1}$) to grow similarly to MV-LeuF1 ($\mu$ = 0.20 ± 0.01 $h^{-1}$). The latter strain did not produce KIC because it carries the native *ilvE* gene. SH-KIC20 produced approximately 19 mM KIC in 49 h, which is comparable to the L-leucine titer achieved with its ancestor strain MV-Leu20. Strain MV-KICF1 accumulated approximately 47 mM KIC after 32 h when supplemented with L-isoleucine, which corresponds to L-leucine production achieved with MV-LeuF1. In contrast, omitting L-isoleucine yielded a maximal KIC concentration of only 34 mM after 95 h. Since IlvE is the main transaminase for the formation of all three BCAAS, accumulation of the other two keto acids KIV and KMV as by-products was expected as well. Indeed, accumulation of KMV and KIV was detected in the supernatants of both KIC producers. The ancestor strain MV-LeuF1 produced more L-valine as by-product than strain MV-Leu20 (Figure 6) so that, as expected, also an increased KIV level could be detected in the respective KIC producer MV-KICF1 in comparison to SH-KIC20. Notably, also L-leucine was detected in supernatants of the KIC producing strains. Strain MV-KICF1 supplemented

with L-isoleucine yielded best values concerning titer (46.7 ± 4.1 mM), yield (0.204 ± 0.018 mol per mol of glucose), and productivity (1.41 ± 0.13 mmol $l^{-1}$ $h^{-1}$).

An interesting observation of the shake flask experiments using the KIC producers was that KIC concentrations decreased with proceeding cultivation time after they had reached the maximal values. Maximal KIC concentration obtained with MV-KICF1 decreased from 46.7 ± 4.1 mM after 32 h to 41.0 ± 3.4 mM after 48 h. This decrease was not observed for L-leucine produced by the respective ancestor production strains. It was assumed that a re-uptake of the produced KIC into the cells from the supernatant occurred and that KIC was taken up by the cells and transaminated, at least partly, to L-leucine. Indeed, L-leucine concentrations in supernatants of MV-KICF1 increased moderately from 3.0 ± 0.2 mM after 32 h to 4.0 ± 0.1 mM after 48 h.

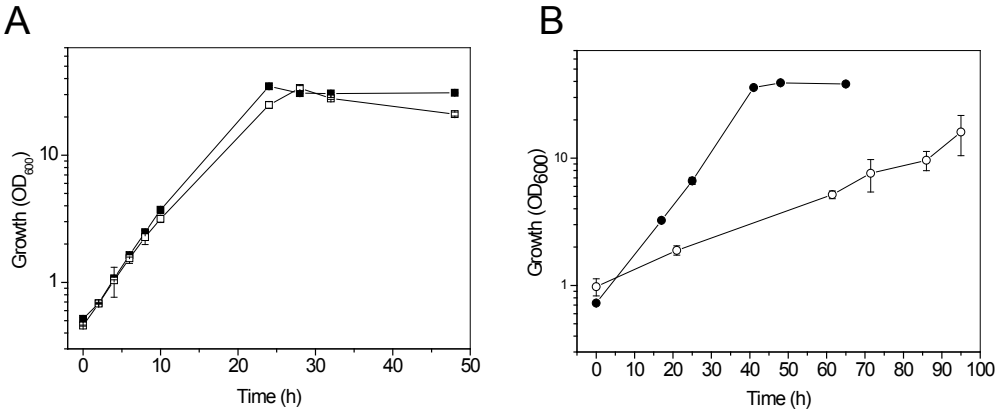


**Figure 11. Growth curves of *C. glutamicum* strains MV-KICF1 and SH-KIC20 in shake flasks with CGXII minimal medium and 4% (w/v) glucose.**

Panel A shows the growth of MV-KICF1 supplemented with 1 mM L-isoleucine (□) and its ancestor strain MV-LeuF1 (■) as reference (inoculated to initial $OD_{600}$ = 0.5). Panel B shows MV-KICF1 without supplements (○) and strain SH-KIC20 without supplements (●) as reference (inoculated to initial $OD_{600}$ = 1).

**Table 12. Results of shake flask cultivations of strains MV-KICF1 and SH-KIC20 including growth rates, maximum KIC and by-product concentrations, as well as specific molar yields and volumetric productivities[a,b].**

| | MV-KICF1 + 1 mM L-isoleucine (32 h) | MV-KICF1 without L-isoleucine (95 h) | SH-KIC20[d] without L-isoleucine (49 h) |
|---|---|---|---|
| Growth rate ($h^{-1}$) | 0.21 ± 0.01 | 0.03 ± 0.01 | 0.08 ± 0.01 |
| KIC (mM) | 46.7 ± 4.1 | 31.8 ± 2.1 | 18.8 ± 0.67 |
| By-products[c]: | | | |
| KIV (mM) | 13.3 ± 2.2 | 19.0 ± 4.1 | 2.6 ± 0.3 |
| KMV (mM) | 8.8 ± 1.3 | 4.9 ± 0.6 | 8.7 ± 0.1 |
| L-leucine (mM) | 3.0 ± 0.2 | 10.3 ± 3.1 | 4.8 ± 0.2 |
| Molar product yield (mol KIC / mol glucose) | 0.204 ± 0.018 | 0.143 ± 0.010 | 0.084 ± 0.001 |
| Volumetric productivity (mmol KIC / (l x h)) | 1.41 ± 0.13 | 0.34 ± 0.02 | 0.38 ± 0.02 |

[a] Cultivations were run with 50 ml CGXII minimal medium with 4% (w/v) glucose. Addition of L-isoleucine is indicated. Cultivation time is shown in parentheses.
[b] The values given are the average data with standard deviations from three independent cultivations.
[c] Concentrations of L-valine and L-isoleucine were below 2 mM.
[d] Cultivation of SH-KIC20 supplemented with 1 mM L-isoleucine was not tested.

### 3.10.3 Batch fermentation of MV-KICF1

To test the possibility for a process scale-up, strain MV-KICF1 was cultivated in bioreactors in batch mode (Figure 12). L-Isoleucine (0.3 g $l^{-1}$) was added to the fermentation medium because this strain performed best when supplemented with this amino acid.

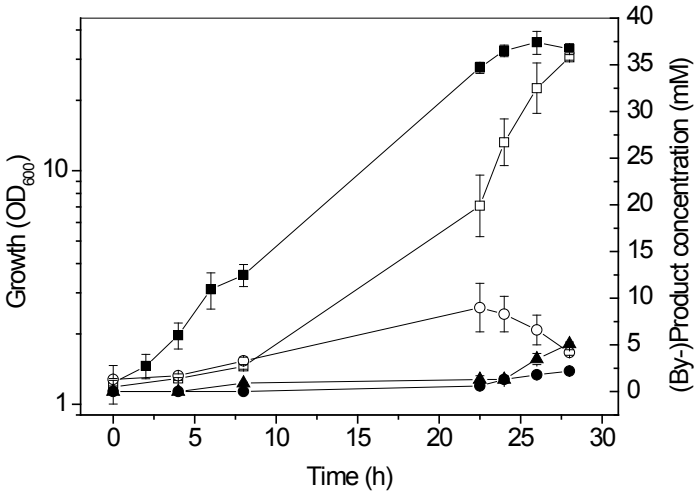


**Figure 12. Batch fermentation of MV-KICF1.**

Growth (■) as well as formation of KIC (□), KMV (●), KIV (○), and L-leucine (▲) is shown. Strain MV-KICF1 was cultivated in Multifors bioreactors filled with 500 ml modified CGXII medium containing 4% (w/v) glucose and 0.3 g $l^{-1}$ L-isoleucine. The pH was kept constant at value 7.0 by automatic addition of 3 M HCl or 3 M NaOH. The growth rate was 0.14 ± 0.01 $h^{-1}$. No L-isoleucine was detected after 28 h and L-valine concentration was below 1.5 mM. The data represent mean values and standard deviations obtained from three independent fermentations.

The batch fermentations revealed a production pattern similar to that obtained in shake flask cultivations, although lower concentrations of product and by-products were obtained. MV-KICF1 produced approximately 35.8 ± 0.3 mM KIC. By-products were 2.2 ± 0.1 mM KMV, 4.2 ± 0.3 mM KIV, and 5.1 ± 0.2 mM L-leucine, which meant that the keto acid concentrations were clearly decreased in comparison to shake flask cultivations while L-leucine concentrations were slightly increased.

Interestingly, additional feeding of 50% (w/v) glucose resulted in maximal KIC concentrations of approximately 50 mM without further increasing. This was accompanied by an arrest of growth of the cells and no further consumption of the added glucose (data not shown). Possibly, an inhibition of cell metabolism due to elevated KIC concentrations occurred.

# 4 Discussion

L-Leucine is an interesting product with diverse commercial applications explaining the demand for strains that can be used for the ecological and economically competitive biotechnological production of this amino acid. In this study, potent L-leucine producing *C. glutamicum* strains were constructed by rational design, which achieved high values in terms of product titer, yield, and volumetric productivity as well as only low by-product formation. An important characteristic of these strains is the absence of auxotrophies, heterologous genes, and plasmids, thus preventing conflicts with regulatory restrictions and avoiding the use of cost-intensive supplements and antibiotics. These properties make these strains highly suitable for industrial application.

Moreover, the feasibility of easily turning L-leucine production strains into producers of the precursor 2-ketoisocaproate (KIC), which also has interesting applications in medicine and other fields, was shown.

## 4.1 IPMS is the key enzyme for L-leucine production

This work started with the weak L-leucine producing strain B018, which had been created by random mutagenesis. The aim was to identify the genes responsible for L-leucine accumulation by comparison of genomic data, in this case DNA sequences related to L-leucine biosynthesis of B018 with respect to the wild-type strain. The identified target genes were supposed to be transferred into the wild type background to create a genetically defined and minimally mutated strain. This strategy of “genome-based strain reconstruction” was already successfully employed for L-lysine production with *C. glutamicum* (Ohnishi et al., 2002). This approach revealed that B018 carries two amino acid exchanges in its *leuA*-encoded 2-isopropylmalate synthase (IPMS) with respect to the wild-type sequence. IPMS, the first enzyme in specific L-leucine biosynthesis, emerged to be a key player enzyme for L-leucine production. The integration of the mutations coding for the respective amino acid exchanges into the *leuA* gene of the wild type (with deleted *ltbR* gene) indeed led to L-leucine production in similar concentrations as achieved with B018. Remarkably, the created strain MV-Leu7 exhibited a significantly higher growth rate than B018 (Table 9), leading to an increased productivity. Strains achieved by random mutagenesis are often inferior to the wild type in properties that are important for a productive industrial application, e.g. growth, sugar consumption, or stress tolerance. This is attributed to genome-wide secondary mutations, which often have detrimental effects (Ohnishi et al., 2002). Therefore, it is advantageous to transfer only beneficial mutations into the wild-type strain by allelic replacement to achieve improved performance and increased robustness (Ikeda et al., 2006). The comparison of the

rationally designed strain MV-Leu7 and B018 achieved by a random approach demonstrates the benefit of this strain reconstruction strategy based on genomic data.

Transcription of *leuA* coding for IPMS is controlled by attenuation and IPMS activity is subject to feedback-inhibition with a $K_i$ for L-leucine of 0.4 mM (Pátek et al, 1994). This mechanism represents the major bottleneck for L-leucine production because it prevents the accumulation of amounts of L-leucine exceeding approximately 1 mM. This tight control of the L-leucine biosynthesis pathway, also in comparison to the other BCAAs, is emphasized by the internal concentrations of the BCAAs in the cell: While there are about 5 mM L-isoleucine and 7 mM L-valine, no L-leucine (concentration < 1 mM) is detected in the wild type of *C. glutamicum* (Leyval et al., 2003). L-Leucine has high metabolic costs due to the need of two molecules of pyruvate and one molecule of acetyl-CoA (altogether eight carbon atoms) to synthesize one molecule of L-leucine (six carbon atoms). It has been reported recently that L-leucine is even the metabolically most expensive amino acid in *E. coli* (Kaleta et al., 2013). The maximal theoretical molar yield of L-leucine per glucose was calculated to be 0.6 mol per mol of glucose in *C. glutamicum* with non-growing cells (Stephan Noack, FZ Jülich, personal communication). In comparison, the theoretical yields of the other BCAAs L-valine and L-isoleucine are higher, i.e. up to 1 mol per mol of glucose (Bartek et al., 2010) and 0.78 mol per mol of glucose (Stephan Noack, FZ Jülich, personal communication), respectively. Due to the high costs of L-leucine biosynthesis, tight regulation of the starting enzyme IPMS is reasonable. Correspondingly, even overexpression of wild-type *leuA* yielded only very low L-leucine titers in the supernatant. In contrast, overexpression of *leuA* from strain B018 led to L-leucine accumulation of up to 40 mM. The two amino acid exchanges that were observed in IPMS of strain B018 in comparison to the wild-type sequence are located in the putative binding pocket for L-leucine formed by three amino acid residues, which were deduced from the crystal structure of the homologous IPMS of *M. tuberculosis* (Koon et al., 2004). The exchange of the corresponding residues in IPMS of *Saccharomyces cerevisiae,* which had been identified by analysis of 5,5,5-trifluoro-DL-leucine resistant mutants, led to a loss of feedback-inhibition (Cavalieri et al., 1999). Indeed, also the simultaneous exchange in the three corresponding residues of the corynebacterial protein abolished feedback-inhibition (IPMS_mod). IPMS of *M. tuberculosis* is a homodimer and each monomer consists of a catalytic domain that is connected to a regulatory domain including the L-leucine-binding pocket (Koon et al., 2004). Huisman et al. (2012) showed that the removal of the C-terminus (regulatory domain) of the mycobacterial protein disrupts the functional substrate binding. The truncated variant is able to bind KIV, but cannot catalyze the reaction between KIV and acetyl-CoA. Although the catalytic and regulatory parts of the protein represent separate domains, they obviously influence each other. Therefore, Huisman and co-workers assumed that the regulatory domain is crucial for ensuring protein dynamics to provide efficient

catalysis. The homologous IPMS of *C. glutamicum* exists also as a homodimer (Pátek et al., 1994) and, due to its sequence similarity, it can be assumed that it shares similar characteristics concerning its structure and reaction mechanism with the mycobacterial enzyme. IPMS from B018 exhibited exceptional kinetic characteristics since its activity was comparable to the wild-type enzyme, but its feedback-inhibition was completely lost. Similar mutations were identified in the aspartate kinase of *C. glutamicum*, resulting in enzyme activities comparable to the wild-type enzyme, but it was not inhibitable by L-lysine plus L-threonine (Chen et al., 2011). In contrast to IPMS_B018, the amino acid exchanges of IPMS_mod obviously negatively affect the enzymatic abilities of the catalytic domain. This suggests an influence of the regulatory domain on the catalytic domain as it was shown for the mycobacterial homolog.

The most significant step to achieve high L-leucine productivity was to reach a high expression of *leuA*_B018. For the construction of a plasmid-free producer strain, the expression of *leuA*_B018 was increased by replacement of the native promoter and attenuation region with the strong *tuf* promoter and by integration of up to three copies of this $P_{tuf}$-*leuA*_B018 module into the *C. glutamicum* genome. A significant increase in expression of *leuBCD* was achieved by deletion of the transcriptional repressor LtbR (Brune et al., 2007) and contributed to increased L-leucine accumulation.

## 4.2 Increasing precursor supply, detecting further limitations, and performing transcriptomics

Acetohydroxyacid synthase (AHAS) was identified as key enzyme for the production of L-valine (Leyval et al., 2003) because its overexpression or the abolishment of its feedback-inhibition mechanism leads to increased L-valine production (section 1.4). Like many other enzymes that represent the starting point of a specific biosynthesis pathway, this enzyme is inhibited by its end-products, in this case the three BCAAs (Eggeling et al., 1987). Since its inhibition does not exceed 57%, this enzyme is not as tightly inhibited as IPMS and is still enzymatically active even in the presence of high BCAA concentrations so that L-leucine formation is not prevented. Nevertheless, preventing the feedback inhibition of AHAS by mutating its *ilvN*-encoded regulatory subunit (Elišáková et al., 2005) resulted in increased precursor supply and L-leucine accumulation. This step also led to an increase of the by-product L-valine that had to be addressed. The reduction of by-product formation is a crucial factor in strain design and was also a focus of this work because large accumulation of by-products decreases the product yield and increases the costs of purification and recovery of the desired compound (Park et al., 2008). Reduction of L-valine formation was mainly achieved by increasing the activity of feedback-resistant IPMS using multiple copies

of the construct $P_{tuf}$-*leuA*_B018 to redirect KIV to L-leucine formation instead of L-valine synthesis. Remarkably, the integration of *leuA*_B018 under control of the *tuf* promoter yielded higher L-leucine concentrations (55 mM) in a strain with deleted *ltbR* and feedback-resistant AHAS than plasmid-based overexpression of *leuA*_B018 in the same strain background (45 mM), although IPMS activity was about 3-4-fold higher with the latter approach. There are reports in literature that a moderate expression of genes can be more beneficial than plasmid-based overexpression because the latter can, for example, lead to non-physiologically high accumulation of intermediates, which can have toxic and detrimental effects (Reinscheid et al., 1994).

Plasmid-based expression in MV-Leu55 of *leuCD*, *leuB* and *ilvE* revealed no further limitations in the respective enzymatic activities. Besides that, the transport systems for L-leucine were also addressed. Neither plasmid-based expression of *brnFE* nor the deletion of *brnQ* yielded higher product concentrations. The activation of *brnFE* expression encoding the exporter of the BCAAs and L-methionine was previously shown to occur upon increased cytoplasmic concentrations of these amino acids via the transcriptional activator Lrp (Kennerknecht et al., 2002; Lange et al., 2012; Mustafi et al., 2012) and was obviously sufficient for effective L-leucine export. Furthermore, L-leucine is not lost from the supernatant by re-uptake via the *brnQ*-encoded importer. In contrast, overexpression of *brnFE* and deletion of *brnQ* was beneficial for the production of L-isoleucine (Xie et al., 2012).

Genome-wide expression analysis verified increased expression of genes responsible for L-leucine biosynthesis and proved the success of the metabolic engineering steps performed. Increased expression of *leuCD*/*leuB* and *brnFE* was attributed to the deletion of *ltbR* and activation via Lrp due to increased internal BCAA concentrations, respectively (see above). Additionally, cg3022 appeared as the highest up-regulated gene when L-leucine was produced. The Cg3022 protein is annotated as an putative acetyl-CoA acetyltransferase and shares significant sequence similarity to the C-terminal regulatory domain of IPMS. Deletion of cg3022 had no influence on L-leucine biosynthesis, but led to higher L-valine accumulation of the respective strain (MV-Leu55 Δcg3022), which could be a hint for Cg3022 being connected to L-valine biosynthesis or its regulation. Further investigations are necessary to unravel the function of Cg3022. It may represent a novel target for optimization of L-valine biosynthesis. This employed DNA microarray-based strategy represents a useful tool to identify novel target genes and was already used for improving L-lysine production with *C. glutamicum* by Sindelar and Wendisch (2007).

### 4.3 Balancing titer, yield, and productivity

The production of L-leucine is performed in a growth-coupled process. The biosynthesis of the BCAAs is closely connected to the central metabolism by using pyruvate as a substrate so that both biomass and product formation influence the overall productivity. That means that the flux of the glucose-derived precursor pyruvate splits up into biomass and product, therby preventing the simultaneous maximization of growth yield and product yield (Zhuang et al., 2013). Due to the high metabolic costs of two molecules of pyruvate and one molecule of acetyl-CoA for the synthesis of one molecule of L-leucine, growth rates significantly decrease with increased product concentrations (section 3.8). Reports dealing with the modeling of bioprocesses to achieve optimal results (Anesiadis et al., 2008; Zhuang et al., 2013) came to the conclusion that the three factors yield, productivity, and product titer have to be balanced and that this balance is crucial to design an economically viable process. High titer is especially required to reach high product concentrations and for minimizing the costs of separation of product and by-product (Zhuang et al., 2013). High yields from carbon sources (glucose in this work) are desired to achieve as much product as possible from small substrate amounts to reduce costs for feedstock used for fermentation. Also reduced process times for an economic process have to be addressed, which is reflected in high volumetric productivity. Summed up, the goal is to achieve high product titers in a short period of time with high yields to reduce capital as well as operating costs and to facilitate downstream processing. First steps in strain construction in this work aimed at reaching high product titers and yields. Strain MV-Leu55 reached the highest L-leucine titer (55 mM) and yield (0.25 mol per mol glucose) in shake flask experiments, but exhibited a decreasing growth rate after 10 h accompanied by a decreased glucose uptake rate. This led to a rather low volumetric productivity. The assumption that the reduced growth rate might be caused by high L-leucine concentrations could not be confirmed. Lange (2004) showed that L-leucine has only a moderate negative effect on the growth rate even at concentrations of about 100 mM. Besides that, MV-Leu55 was the only strain that exhibited a bi-phasic growth behavior. The reason for this is unclear. Interestingly, a growth behavior similar to that of MV-Leu55 has recently been reported for a KIV producer of *C. glutamicum* (Buchholz et al., 2013). Another notable observation was that this bi-phasic growth did not appear in batch fermentations. It is possible that this effect is due to the constant aeration of the fermentation vessel, which provides sufficient oxygen supply preventing the culture to become anaerobic. However, this does not explain why only MV-Leu55 exhibited bi-phasic growth in shake flask cultivation. Possibly, the high L-leucine production load makes the strain more sensitive to the limited aeration as characteristic for shake flask cultivation. It was reported by Varma and Palsson (1994) that appropriate control of oxygen supply can enhance population stability of engineered L-valine, L-tryptophan, and L-lysine production strains.

The glucose uptake rate of strain MV-Leu55 could be increased by 40% by deletion of the *iolR* gene, which leads to the derepression of *iolT1* encoding a *myo*-inositol transporter which also catalyzes glucose uptake (Ikeda et al. 2011, Klaffl et al. 2013; Lindner et al. 2011). Glucose imported by IolT1 is phosphorylated by an ATP-dependent glucokinase (*glk*, cg2399; Park et al. 2000) or by a polyphosphate/ATP-dependent glucokinase (*ppgK*, cg2091; Lindner et al. 2010) and channeled into central metabolism. Deletion of *iolR* in MV-Leu55 resulted in a slightly increased and constant growth rate that led to a shortened cultivation time and a 40% increase of volumetric productivity. Surprisingly, the introduction of the third $P_{tuf}$-*leuA*_B018 copy, which failed in strain MV-Leu55, was easily achieved in strain MV-Leu55 Δ*iolR*. The reason for this difference is not known. It might be related to the presence of the PTS-independent glucose uptake system IolT1, but other IolR target genes that are derepressed could also play a role. Interestingly, Ikeda (2012) obtained data indicating that the employment of a non-PTS-dependent glucose transport system or co-utilization of the PTS and the non-PTS route for glucose uptake, as it is the case for MV-Leu55 Δ*iolR,* could increase cell robustness in large-scale industrial fermentation processes.

Strain MV-LeuF1, which carries three $P_{tuf}$-*leuA*_B018 copies, produced significant concentrations of L-valine, which could be diminished by increasing the supply of acetyl-CoA by reduction of citrate synthase expression via a promoter exchange (van Ooyen et al., 2012). The resulting strain MV-LeuF2 exhibited the highest volumetric productivity so far. These results demonstrate that a balanced precursor supply and moderate increase of enzyme activities is a very efficient way to redirect the metabolic flux at important branch points.

### 4.4 Fed-batch fermentations

In fed-batch cultivations, a pH of 6.5 turned out to be favorable for the production of L-leucine with strains MV-LeuF1 and MV-Leu2. Growth rates of MV-LeuF1 at pH 6.5 and 7.2 were similar, which is in line with the findings of Follmann and colleagues (2009) that effective pH homeostasis in *C. glutamicum* takes place between pH values from 6 to 9. Interestingly, MV-LeuF1 stopped growing at an $OD_{600}$ of 5-6 when cultivated at pH 8.0. One possible explanation is a detrimental internal accumulation of amino acids due to a preferential uptake of BCCAs in comparison to excretion. The optimum pH for the uptake of L-isoleucine was reported to be 8.5 (Ebbighausen et al., 1989). Nevertheless, as data for the pH optima for uptake and excretion of L-valine and L-leucine are lacking, further investigation is necessary to explain the effect of the pH value on the L-leucine production process.

Despite increased L-lysine concentrations, an important advantage of pH 6.5 was a reduction of the by-products L-valine and L-alanine. Since L-leucine, L-valine, and L-alanine share almost the same isoelectric point, the industrial recovery process becomes very difficult when the latter two amino acids accumulate to high amounts (Hou et al., 2012). Consequently, both strain design as well as fermentation process performance contribute to by-product minimization.

Using optimized cultivation conditions in the bioreactor, a balance of the three crucial factors titer, yield, and productivity was achieved. The highest molar product yield of 0.3 mol L-leucine per mol of glucose was obtained with strain MV-LeuF2. This corresponds to 50% of the theoretical maximal yield (calculated to be 60%) with non-growing cells of *C. glutamicum*. MV-LeuF2 exhibited a maximal growth rate of 0.2 $h^{-1}$, which is about 50% of the growth rate of the wild type under identical conditions. The maximum volumetric productivity in the feed phase was 4.3 mmol $l^{-1}$ $h^{-1}$ and maximal L-leucine titers exceeded 180 mM. Values were calculated up to the point where precipitation of L-leucine was observed because only the soluble L-leucine fraction was taken into account for calculating yield and volumetric productivity. Precipitation of L-leucine above approximately 150 mM has two benefits: firstly, it simplifies downstream processing and, secondly, an insoluble product has no osmotic effect on the cells, which is helpful to prolong the fermentation process. Strain MV-LeuF2 achieved a balance of titer, yield, and productivity and reached the highest values for these three factors reported so far for a bacterial L-leucine production strain cultivated in a defined minimal medium. Besides that, MV-LeuF2 is prototrophic, genetically defined, and does not carry plasmids. All these aspects make it highly interesting for industrial application. In contrast, previously described L-leucine-production strains of *C. glutamicum* were generated by random mutagenesis and contained amino acid auxotrophies. Although these strains reach comparable L-leucine titers (>20 g $l^{-1}$), their cultivation requires the addition of the corresponding amino acids to the cultivation medium, which increases the production costs (Ambe-Ono et al., 1996). As already mentioned, there are only few works dealing with L-leucine production and even no report about a rationally designed *C. glutamicum* strain. Only *E. coli* strains were engineered rationally for L-leucine production, but they did not reach performances comparable to the strains in this work (Park and Lee, 2010). In contrast, several reports concerning L-valine and L-isoleucine production using *C. glutamicum* are described and selected production values are given in the following.

L-Valine titers of 410 mM with maximum yields of 0.75 to 0.86 mol per mol of glucose were reported for aerobic fed-batch fermentations at high cell densities (Blombach et al., 2008) and 1280 mM with yields up to 0.88 mol per mol glucose were obtained under oxygen-deprived conditions (Hasegawa et al., 2013). Based on a theoretical yield of 1 mol per mol of glucose, these values demonstrate the availability of high-performance *C. glutamicum*

production strains for this BCAA. Also a variety of L-isoleucine producers exist. Considering a maximal theoretical yield of 0.78 mol of L-isoleucine per mol glucose, titers up to 234 mM L-isoleucine with a yield of 0.165 mol per mol of glucose (Yin et al., 2012) are comparably low. Despite the fact that a direct comparison of the BCAA production strains is not possible due to different factors such as process design, theoretical yields, and solubility, values reached with the L-leucine production strains created in this work are satisfying, but can be certainly improved. A great advantage of rationally designed strains is the possibility to optimize them further in comparison to strains obtained by random mutagenesis, which often result in "dead-end" strains (section 1.4). Possible improvements of L-leucine production are discussed in section 4.6.

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### 4.5 Production of 2-ketoiscocaproate

The keto acid 2-ketoisocaproate (KIC), the direct precursor of L-leucine, represents an interesting commercial product due to its applications in different fields (section 1.2). Since it is mainly produced chemically, the biotechnological production is an interesting alternative. In this work, it was shown that KIC production strains can easily be achieved by deleting *ilvE* in existing L-leucine producers. The gene *ilvE* codes for the enzyme IlvE, which is the main transaminase for the conversion of the keto acids KIC, KIV, and KMV into L-leucine, L-valine, and L-isoleucine, respectively, in an L-glutamate dependent manner (Radmacher et al., 2002). IlvE has rather high affinities for its three substrates, with $K_M$ values of 0.15 mM, 0.63 mM, and 0.23 mM for KIC, KIV, and KMV, respectively (Marienhagen et al., 2005). Correspondingly, the deletion of *ilvE* leads to auxotrophies for L-leucine and L-isoleucine in the wild-type strain, but not for L-valine because transaminase AvtA exhibits high activity and affinity ($K_M$ = 2.51 mM) for the formation of L-valine from KIV in an L-alanine dependent manner (Marienhagen et al., 2005). Besides that, other aminotransferases such as AroT have weak side activities for L-leucine formation. This is also true for AvtA exhibiting a rather weak affinity for KIC, with a $K_M$ value of 16.84 mM (Marienhagen et al., 2005). The *C. glutamicum* wild-type strain and also the Δ*ilvE* strain do not accumulate KIC in the supernatant. Since IPMS is strongly feedback-inhibited, even small internal L-leucine concentrations due to the side activities of the other transaminases are sufficient to inhibit IPMS (section 4.1), preventing KIC to accumulate. Even in L-leucine producers, KIC was not detected in the supernatant, which confirms the effective transamination reaction performed by the *ilvE*-encoded transaminase. Correspondingly, transamination reaction by IlvE was not found to be a bottleneck for L-leucine production in this work. Only in fed-batch fermentations without feeding of additional ammonium sulfate, low KIC accumulation occurred, which offers the possibility to produce KIC by limiting the nitrogen concentration.

This work shows that L-leucine production strains can serve as a basis for the production of KIC. Recently, also Haas (2013) confirmed that deletion of *ilvE* in L-leucine producers leads to KIC production and that KIC accumulates to concentrations similar to the L-leucine concentrations obtained with the respective ancestor strains. Since it was intended to design strains devoid of auxotrophies, deletion of *ilvE* was not desired. Therefore, reduction of IlvE activity by exchanging the ATG start codon was used to enable both growth and KIC accumulation. This approach was successful for the tested KIC producers, MV-KICF1 and SH-KIC20, but had the disadvantage of very low volumetric productivities for KIC. Besides that, this approach worked better for the weaker production strain SH-KIC20. This strain reached the expected KIC concentrations of about 20 mM, whereas MV-KICF1 only reached the expected maximal 45 mM KIC when L-isoleucine was added to the medium. Interestingly, addition of L-leucine was neither necessary for SH-KIC20 nor for MV-KICF1. L-Leucine was still detected in significant concentrations as by-product. This observation was also found by Haas (2013) for other KIC producing strains. This can be explained by the fact that KIC accumulates to high internal KIC concentrations so that they reach the $K_M$ values of other transaminases which are able to convert KIC to L-leucine as side activity (see above). Eventually, growth and even accumulation of L-leucine is enabled due to the high KIC concentrations. Since supplementation of L-isoleucine to MV-LeuF1 resulted in similar growth and productivity as its ancestor strain MV-LeuF1, it was confirmed that only L-isoleucine supplementation was necessary for enabling high growth rates.

The start codon exchange was a proof-of-principle approach and showed that downregulation of IlvE activity allows a balance of growth and KIC accumulation without supplementation of BCAAs. To achieve higher productivities, other possibilities are conceivable. Gene expression of *ilvE* could be fine-tuned by using different promoter libraries (Hammer et al., 2006). For example, different variants of *dapA* promoters described by Vašicová et al. (1999) could be employed. These were used for downregulating *gltA* expression by van Ooyen et al. (2012) and this strategy was also used for the construction of L-leucine production strain MV-LeuF2 in this work. Analogously to the optimization of fermentations of L-leucine production strains, process conditions for cultivation of KIC producers in bioreactors should be improved to reach the higher KIC titers obtained in shake flask cultivation. This could be done, for example, by testing different oxygen and pH conditions. Since the latter worked for L-leucine production to reduce by-product formation, optimization of process conditions should also be a possibility to decrease accumulation of the other keto acids KIV and KMV as well as L-leucine in favor of increased KIC production. Moreover, it is obviously important to finish fermentations at the point of highest KIC concentrations to prevent a loss of KIC from the supernatant. It is likely that this was caused

due to re-uptake of KIC by the cells and subsequent transamination to L-leucine as it was already shown by Groeger and Sahm (1987).

Very recently, Bückle-Vallant et al. (2014) published a *C. glutamicum* strain for the production of KIC. The reported strain carried a plasmid and was not designed on the basis of plasmid-free L-leucine producers as shown in this work (section 1.4). With glucose as the sole carbon source, the strain reported by Bückle-Vallant reached KIC titers (54 ± 4 mM) and yields (0.22 mol per mol of glucose) in a comparable range as it was achieved with strain MV-KICF1 when supplemented with L-isoleucine (Table 12).

In contrast to the reported KIV producers reported by Krause et al. (2010) and Buchholz et al. (2013), with titers of approximately 188 mM and 303 mM KIV, respectively, KIC titers shown in this work and that of Bückle-Vallant and co-workers (2014) are rather low. Interestingly, only KIC concentrations up to approximately 50 mM were reached when additional glucose was fed at the end of the batch fermentations with strain MV-KICF1. Besides that, glucose was not fully consumed and cells stopped growing, leading to the assumption that cell growth was inhibited by the elevated KIC concentrations. Bückle-Vallant et al. (2014) indeed found competitive and noncompetitive inhibition of IPMS and IPMD, respectively, by KIC, which provides an explanation of the observed limited KIC production capacities. Also a competitive inhibition of AHAS by KIV was reported by Krause et al. (2010) so that the formation of this keto acid as by-product should be minimized. Therefore, the inhibition mechanisms should be addressed in future work to improve KIC production.

## 4.6 Outlook: Possible improvements for L-leucine and 2-ketoisocaproate production

Besides the performed metabolic engineering steps and optimized process conditions, further aspects should be considered to improve L-leucine production and that of KIC as well. Apart from the pH value, oxygen supply is another parameter that can be addressed to improve strain performance. For example, Hou et al. (2012) showed that by-product formation in L-valine production with *C. glutamicum* can be reduced by different dissolved oxygen concentrations. Similarly, Akashi et al. (1978) reported that L-leucine accumulation can be increased under oxygen-limited conditions. Another possibility would be to cultivate the engineered cells anaerobically to reduce growth but retain metabolic activity. Hasegawa et al. (2013) engineered strains that exhibit high performance concerning titer, productivity, and yield for L-valine formation under oxygen-deprived conditions. Since NADPH is needed as co-factor for L-leucine biosynthesis (Figure 7), the improvement of NADPH supply, for example by overexpression of NAD kinases (Shi et al., 2012), should also be beneficial for L-leucine production. The downregulation of *ilvA* expression coding for threonine

dehydratase could also be beneficial for L-leucine formation because it would reduce the competition of L-isoleucine and L-valine/L-leucine intermediates for the enzymes AHAS, AHAIR, and DHAD (Holátko et al., 2009) (Figure 7). Moreover, an L-isoleucine shortage caused by *ilvA* downregulation could derepress the attenuation of the *ilvBNC* operon (Morbach et al., 2000) or reduce the growth rate in favor of improved L-leucine formation as it was shown for L-valine production (Ruklisha et al., 2007). Another possibility to increase L-leucine production would be the exchange of the promoter of the *ilvBNC* operon including its attenuator sequence against a strong promoter to further increase precursor supply.

All metabolic engineering steps of this work were performed rationally, but were mainly applied to local target genes. Another advanced approach for strain design represents systems metabolic engineering (Figure 13). This strategy provides a system-wide view of the producer cell and has already applied for *C. glutamicum* (Park et al., 2008, Wendisch et al., 2006b). This strategy could be used to improve the existing L-leucine producers by, for example, performing analysis of metabolic fluxes or proteomics. A first step towards this strategy was already done in this work by performing genome-wide expression analysis (transcriptomics) with different L-leucine producers that revealed a possible novel target gene (cg3022).

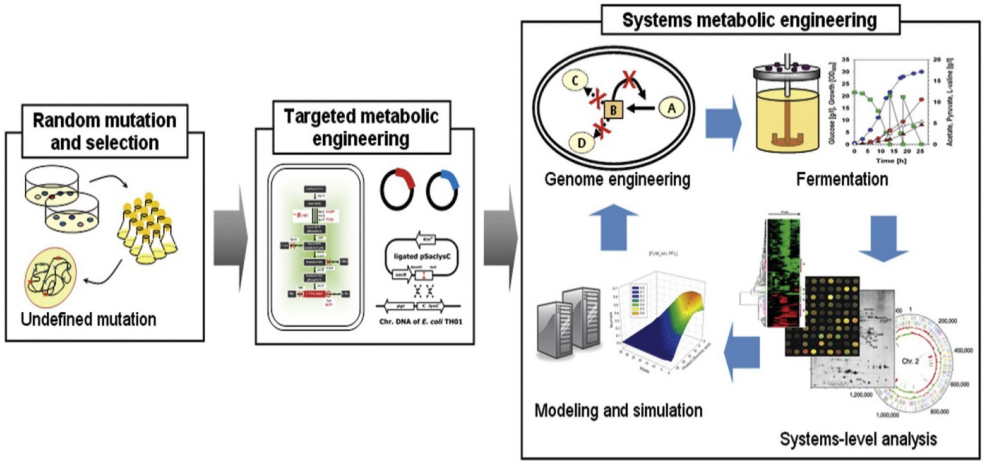


**Figure 13. Systems metabolic engineering for advanced strain development (from Park and Lee, 2008).**

Systems metabolic engineering represents an advancement of random mutagenesis/selection and targeted metabolic engineering. In this approach, systems-level analyses of data obtained by "omics"-technologies (i.e. genome, transcriptome, proteome, metabolome, and fluxome data) and computational analyses are combined with metabolic engineering. Fermentation and downstream processing are considered at early stages of strain design and performed to characterize the engineered strain and to improve it further. The systems metabolic engineering cycle is repeated until a strain with the desired properties is obtained.

Strains MV-LeuF1 and MV-LeuF2 might not only be highly interesting for industrial L-leucine production but additionally for the production of related products. This was shown for the production of KIC in this work. Moreover, similar to the production of isobutanol from KIC (Blombach et al., 2011), the production of isopentanol should be possible from KIC (Cann and Liao, 2010).

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## Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.
Die Dissertation wurde in der vorgelegten oder ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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